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Analysis of Polyethylene Glycol Modified Superoxide Dismutase by Chromatographic, Electrophoretic, Light Scattering, Chemical and Enzymatic Methods

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Covalent conjugation of bovine erythrocyte superoxide dismutase (SOD) with activated polyethylene glycol (PEG) results in a mixture of modified species (PEG-SOD) with properties different from those of the native enzyme. The components of this mixture were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), isoelectric focusing, and chromatographic (size-exclusion, anion-exchange, cation-exchange and reverse-phase high performance liquid chromatography) techniques. Physicochemical properties such as apparent molecular weight, isoelectric point, relative hydrophobicity and relative cation–anion charge number were measured by electrophoretic and chromatographic procedures. Dispersity and apparent radius were examined by chromatographic and light scattering techniques. The extent of covalent modification and enzymatic activity change were measured by chemical and spectroscopic methods, showing that activity loss was not due to catalytic site modification. The properties of the PEG-modified form of the enzyme were compared with those of native SOD and showed that in addition to changing biological properties, PEG modification of proteins can result in a product with unexpectedly high heterogeneity and substantial changes in isoelectric point and hydrophobicity. Altered biological properties may therefore not merely be due to shielding of protein surface by PEG chains. Apparent properties of PEG modified proteins such as molecular weight were found to be highly method dependent, with poor agreement being shown among classical measurements.

Keywords——superoxide dismutase; polyethylene glycol modified protein; chromatography; electrophoresis; light scattering; isoelectric focusing

Introduction

Bovine erythrocyte superoxide dismutase (SOD) is currently under active investigation as a possible anti-inflammatory drug and as a protectant during reperfusion of ischemic tissue.[1-3] Limited plasma half life and the ability to elicit immunogenic and antigenic responses in humans limits the potential utility of this therapeutic agent. Modification of SOD and other enzymes by covalent attachment of polyethylene glycol (PEG) results in species with dramatically increased plasma half lives and very low immunogenicity.[4-8] Several studies have been performed by developers of polyethylene glycol modified SOD (PEG-SOD) to examine the extent of structural changes which occur upon modification, and resultant changes in enzymatic and pharmacokinetic properties and dispersity.[7,9,10] Changes in size-exclusion and anion-exchange chromatographic properties have also been noted.[4,5,7,9]

No comprehensive examination of heterogeneity, or characterization of changes in SOD physicochemical properties due to PEG modification have been performed. Furthermore, the effect of covalently attached random coil hydrophilic polymers such as PEG on classical protein separation and analytical techniques (size–exclusion, ion-exchange, and reverse-phase chromatography, electrophoresis, isoelectric focusing, and photon correlation spectroscopy), and physicochemical measurements (molecular weight) has not been addressed.

This paper examines the heterogeneity and activity of PEG-SOD preparations, and compares physicochemical properties (size as measured by apparent molecular weight, isoelectric point (pI), and solubility as measured by ion exchange and reverse-phase chromatographic retention times) of the different PEG-SOD species present in a given preparation, with those of native unmodified superoxide dismutase. Since the effect of PEG modification on bioanalytical techniques generally performed in the course of protein characterization is unknown, consequences of PEG modification on measurements commonly performed on proteins were examined by comparing apparent molecular weights determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), size-exclusion chromatography and photon correlation spectroscopy, with the true molecular weight (MW, dalton (Da)) obtained by titration of remaining free amines in the modified enzyme.

Materials and Methods

Chemicals——Bovine erythrocyte SOD (lot No. 10594721 IL/JUL 88) was purchased, in lyophilized form from Boehringer-Mannheim Biochemicals (Indianapolis, IN), as were several of the molecular weight markers used for calibration of the size exclusion columns. PEG-modified bovine erythrocyte SOD modified by attachment of hydroxy succinimide activated PEG (lot No. PSOD86J 2612) was purchased from Enzon Inc. (S. Plainfield, NJ). Trinitrobenzene sulphonic acid (TNBS) was obtained from the Kodak Corp (Rochester, NY). Latex microspheres used for calibrating the photon correlation spectroscopy (LALLS) equipment were purchased from Duke Scientific Corp. (Palo Alto, CA). Various polyethylene oxide molecular weight standards were purchased from Varian (Sunnyvale, CA). Native protein molecular weight markers and pyrogallol were purchased from Sigma Chemical Co. (St. Louis, Mo). Coomassie brilliant blue R-250 staining reagent and SDS were obtained from the Bio-Rad Corp. (Richmond, CA). Precast polyacrylamide plates with a pH range of 3.5 to 9.5 (Ampholine PAG 3.5—9.5) used for isoelectric focusing, were obtained from LKB (Paramus, NJ). Isoelectric point standards and SDS-PAGE protein molecular weight markers and PhastGel Blue 250-R staining reagent were purchased from Pharmacia (Piscataway, NJ). Commercially available 0.45 mm PhastGel 10—15 precast SDS-PAGE gels having a 13 mm stacking gel zone and a 32 mm gradient gel zone (consisting of a continuous 10—15% gradient with 2% cross-linking), were also purchased from Pharmacia. All other reagents, solvents and buffers were of the highest quality available.

Chromatographic Equipment——All high performance liquid chromatographic (HPLC) analyses were performed on a chromatographic system from Waters Associates (Milford, MA) The system consisted of the following components; two 510 pumps, a WISP autosampler with cooling module, a 680 automated gradient controller and a column heater. Proteins were detected by a 410 differential refractometer and a M-490 programmable multi-wavelength UV-vis detector. All results were processed using a Linear dual pen strip chart recorder and a Hewlett Packard-3392A integrator (Palo Alto, CA). Micropak TSK-3000-SW size exclusion columns were obtained from Varian (Sunnyvale, CA). A Vydac TP Silica C4 reverse-phase column was purchased from the Separations Group (Hesperia, CA). Anion and cation ion-exchange columns (SynChropak AX-300, CM-300) were obtained from SynChrom Inc. (Linden, IN).

Electophoresis Equipment——Isoelectric focusing of PEG-SOD was carried out on a horizontal LKB Multiphor II electrophoresis system. Power was supplied by a Macrodrive 5000 power supply. SDS-PAGE experiments were carried out on a Pharmacia Phast System using commercially available PhastGel gradient media. Plates were scanned with an LKB Ultroscan 2222 laser densitometer to determine the positions of native and modified SOD, and the marker proteins.

Laser Light Scattering——Change in molecular diameter of SOD upon PEG modification was determined using a Coulter N4MD LALLS submicron particle size analyzer (Hialeah, FL), employed as a photon correlation spectrometer. Apparent diameter measurements were calibrated using both latex microspheres of known size (Duke Scientific) and polyethylene oxide molecular weight standards (Varian).

Optical Spectroscopy and Enzyme Assays——Absorption spectra of native and PEG-modified SOD were obtained using a Hewlett Packard-8450-A diode array spectrophotometer. Assays were performed (at 420 nm) utilizing the above spectrophotometer. The activities of native and modified enzyme were kinetically determined by the pyrogallol autoxidation method.[11]

Determination of Molecular Weight——TNBS Titration: Using the TNBS method[12] readily accessible free amine residues of the modified and unmodified enzyme were determined colorimetrically at 335 nm. Loss of free amines in the PEG-enzyme is a measure of the number of PEG chains of known MW which have been attached to the protein *via* amino conjugation.

Size-Exclusion Chromatography: The molecular weight of PEG-modified SOD was determined by size-exclusion chromatography using an isocratic mobile phase consisting of 0.05% sodium azide in water at a flow rate of 1.0 ml/min. Optimal results, in terms of separation of components of PEG-SOD, were obtained by using two TSK-3000SW columns (9.4 × 300 mm) in series. Calibration of the column system was performed with the following marker proteins: α-lactalbumin, 14200 Da; chymotrypsinogen A, 25000 Da; SOD, 32000 Da; chicken albumin, 45000 Da; bovine serum albumin and dimer, 66000 and 13200 Da respectively; β-amylase, 200000 Da and blue dextran 2000000 Da. All marker proteins were prepared at *ca.* 1.0 mg/ml in aqueous solutions and injected as 25 μl samples. Detection was by ultraviolet (UV) at 280 nm and by refractive index. Molecular weights of PEG-SOD species were obtained from plots of retention time *vs.* log MW of the marker proteins.

SDS-PAGE: Acrylamide gel electrophoresis of SOD was carried out on a Pharmacia Phast system at a uniform temperature of 15 °C. The system was programmed to apply a constant power of 3.0 W, 250 V and 10 mA for a 70 volthour time period. Precast 0.45 mm PhastGel 10—15 gels were employed in all experiments. All gels were Coomassie stained. Molecular weights of PEG-SOD species were obtained by comparing relative migrations with a plot of migration distances *vs.* log MW determined using protein molecular weight markers (see Table I).

Chromatography——Reverse-Phase Chromatography: Both the unmodified and FEG enzymes were successfully chromatographed using a Vydac C_4 column and a two component gradient mobile phase consisting of: A) 0.1% phosphoric acid in water; B) 0.1% phosphoric acid in acetonitrile–water (95 : 5). The gradient was varied linearly from 25—100% B at a constant flow rate of 1.5 ml/min. Using 100 μl injections of aqueous solutions of SOD and PEG-SOD (1.0 and 1.8 mg/ml, respectively) allowed a straightforward measurement of peaks at 254 nm, a wavelength corresponding to a SOD absorbance maximum.

Anion-Exchange Chromatography: A Synchropak AX-300 weak-anion exchanger column (250 × 4.6 mm) was employed at a pH above the pI's of the enzymes examined. Chromatography was performed using a gradient mobile phase system, at a flow rate of 1.0 ml/min, consisting of A) 0.02 M Tris, pH 7.5; B) 0.02 M Tris, pH 7.5, 0.5 M sodium chloride, which varied from 0—100% B over 10 min. Resolved peaks were measured at 254 nm after 25 μl injections of aqueous enzyme solutions, at the above mentioned concentrations. Under these conditions we were able to elute both the native and modified enzyme.

Cation-Exchange Chromatography: Using the Synchropak CM-300 weak-cation exchange column (100 × 4.6 mm) which uses a macroporous spherical silica bonded polyamide coating derivatized with carboxymethyl groups, it was found that substantially different mobile phase compositions were required for successful elution of SOD and PEG-SOD. Analysis of native enzyme employed a gradient mobile phase system at a flow rate of 1.0 ml/min consisting of A) 0.02 M sodium acetate, pH 5.0; B) 0.02 M sodium acetate, pH 5.0 plus 0.5 M NaCl which varied from 0—100% B over 15 min. An injection volume of 25 μl (1.0 mg/ml) and detection at 254 nm were used. Elution of the PEG-SOD was achieved with an isocratic mobile phase which consisted of 0.001 M sodium acetate, pH 5.0 at a flow rate of 1.0 ml/min. Injection volume was 100 μl (1.8 mg/ml) and detection was by UV at 254 nm.

Electrophoresis——Determination of pI of PEG-SOD by Isoelectric Focusing: Isoelectic focusing of both SOD and PEG-SOD was carried out on a horizontal LKB Multiphor II electrophoresis system at a constant temperature of 10 °C.

A constant power of 50W was maintained using a Macrodrive 5 power supply. Voltage (2000 V) was applied for 1.2 h at a current of 25 mA. Commercially available LKB PAG isoelectric focusing plates having a pH gradient of 3.5—9.5 were used. Bands were visualized by Coomassie staining.

Results

Apparent Molecular Diameter——Native unmodified SOD is too small (<3 nm) to allow measurement by photon correlation spectroscopy (LALLS), as shown in Fig. 1. We were therefore unable to directly compare its molecular size with that of the modified protein. PEG-SOD on the other hand exhibited an apparent molecular diameter of 13.2 nm (measured at a scattering angle of 29.8 °), as shown in Fig. 2. Comparison with apparent diameters of polyethylene oxide standards[13] places the modified enzyme in the size range of a random coil polymer of approximately 95000 Da.

Absorption Spectra——Ultraviolet spectrophotometry of SOD and PEG-SOD showed no perceptable difference in spectra, especially in the case of the band (~258 nm) indicative of copper-imidazole interactions at the active site. The spectra obtained were essentially identical to established characterizations.[14]

Enzyme Assay——A kinetic assay involving SOD mediated supression of pyrogallol autoxidation[11] was used to determine enzyme activities of the native and modified species. A comparison of equal molar concentrations of functional protein (as determined from the

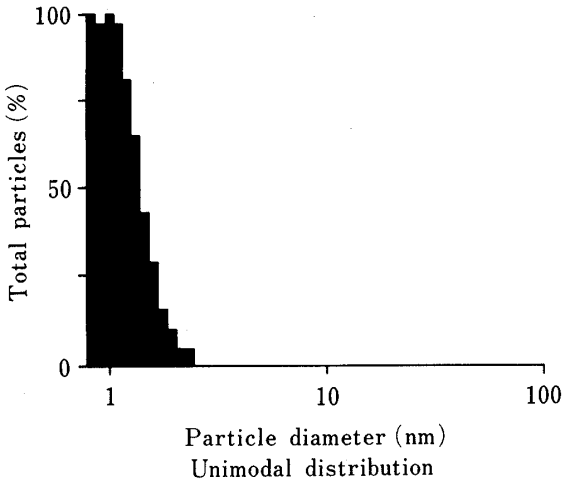


Fig. 1. Unimodal Particle Size Distribution of SOD Obtained by Photon Correlation Spectroscopy of a 1.8 mg/ml Sample in 0.03 M NaCl, 0.03 M Phosphate pH 7.3, at 20.0 °C

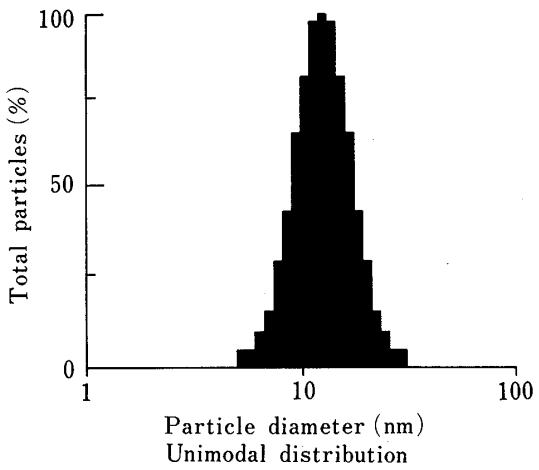


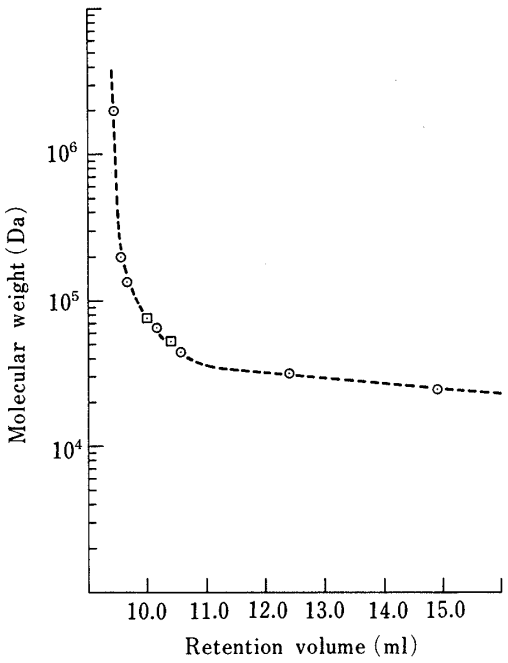
Fig. 2. Particle Size Distribution of PEG-SOD by Photon Correlation Spectroscopy, under Conditions Described in Fig. 1

absorbance of active site copper at 258 nm) showed that the batch (PSOD-86J 2612) of PEG-SOD studied had an activity (inhibition of ΔA_{420}/min) of 46.8% that of the native. It has been previously shown that PEG modification of seven or more SOD lysines results in loss of activity of approximately 50% relative to the native enzyme.[5-7]

Determination of Molecular Weights

TNBS Titration——The average molecular weight of PEG modified SOD can be estimated by determining the number of polyethylene glycol chains which have been covalently attached to a molecule of the native enzyme. The modification occurs primarily at residues possessing readily accessible nucleophilic amines. It has been shown that eighteen to twenty lysine residues of bovine erythrocyte SOD can be covalently modified by activated PEG.[6] The number of amine (lysine) side chains to which PEG chains have been attached can be measured by a comparative titration of free lysines in the native and modified proteins, using trinitrobenzene sulfonic acid.[6,12] Assuming that twenty amines (lysines) are available for modification by TNBS in native SOD,[5] the particular batch of PEG-SOD employed in this study (PSOD-86J 2612) possessed 8.56 ± 0.21 ($n=6$) free amine groups capable of reacting with TNBS. Therefore, an average of 11.4 polyethylene glycol chains have been attached to the batch of SOD under investigation. PEG of an average molecular weight of 5000 was employed in modification of the above batch (personal communication from ENZON Inc.) giving the product an average nominal molecular mass of 86000 Da.

Size-Exclusion Chromatography——Calibration of the two tandem Toyo Soda TSK-3000SW size exclusion columns employed in this study with native globular proteins resulted in the elution volume *vs.* log MW relationship shown in Fig. 3. Separation of PEG-SOD samples on these columns resulted in chromatograms such as the one shown in Fig. 4. A size-exclusion chromatogram of unmodified SOD is shown in Fig. 5 for comparative purposes. Covalent addition of PEG to SOD resulted in a heterogeneous product as evidenced by the two partially resolved broad bands in Fig. 4. The retention times of these peaks of 10.40 and approximately 10.0 min is substantially shorter than that of the native enzyme (12.33 min), and correspond to species possessing molecular masses of 52000 and approximately 75000 Da (Fig. 3). The molecular weight of unmodified bovine erythrocyte SOD is 31300.[15] An attempt to use a calibration curve derived from random coil polyethylene oxide polymer standards to estimate PEG-SOD molecular weight failed, as the PEG-SOD eluted before the void volume



Protein	MW (Da)	t_R (min)
Chymotrypsinogen-A	25000	14.90
Superoxide dismutase	32000	12.35
Chicken albumin	45000	10.56
Bovine serum albumin	66000	10.18
dimer	132000	9.63
β-Amylase	200000	9.56
Blue dextran	2000000	9.45
PEG-SOD No. 1	~75000	10.00
PEG-SOD No. 2	~52000	10.40

Fig. 3. Molecular Weight Calibration Curve for Two Tandem TSK-3000SW Size-Exclusion Columns, Employing Conditions Described in Fig. 4

⊙, native protein standards; □, PEG-SOD peaks.

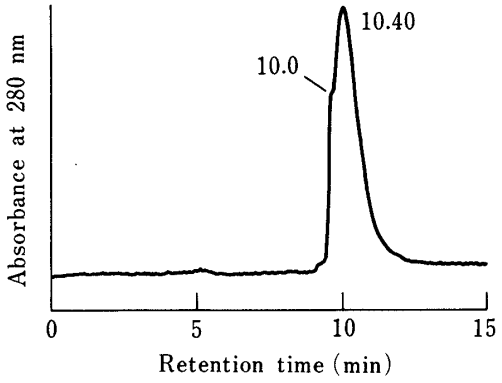


Fig. 4. Size-Exclusion Chromatogram of PEG-SOD Obtained Using Two Tandem TSK-3000 SW Columns, Obtained *via* Elution of 25 μg Samples with 0.05% Sodium Azide at 1.0 ml/min.

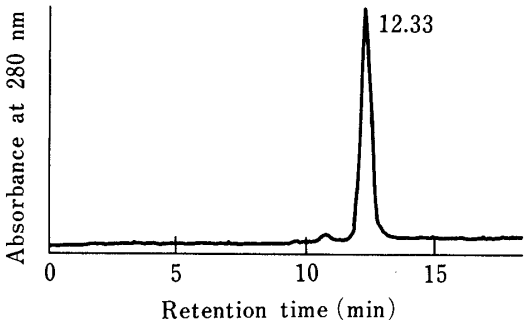


Fig. 5. Size-Exclusion Chromatogram of Native SOD, Employing Conditions Described in Fig. 4

for the largest polymer.

SDS-PAGE——Separation of PEG-SOD species and estimation of their molecular weights was performed by SDS-PAGE, using commerically available PhastGel 10—15 plates. The gradient of this gel is designed to give a linear relationship between a protein's migration distance and the log of its molecular weight, over the molecular weight range 10000 to 250000. Resolution of PEG-SOD into at least six bands is shown in Fig. 6, clearly demonstrating that PEG-SOD is heterogeneous in nature. Estimates of molecular weights for the various synthetic isozymes of PEG-SOD can be determined from the observed mobilities (in mm) on a gel calibrated with standard proteins, both sets of results being shown in Table I. Under the denaturing conditions of SDS-PAGE, PEG-SOD migrates as dissociated subunits rather than as an intact dimer, apparent molecular weights calculated from the data of Table I were therefore multiplied by two to give molecular masses of 34000, 50000, 74500, 107500, 132000, and 162500 Da for the observed multiple bands. As a control, the molecular weight of native unmodified SOD was determined by the above procedure and found to be 33000, in close

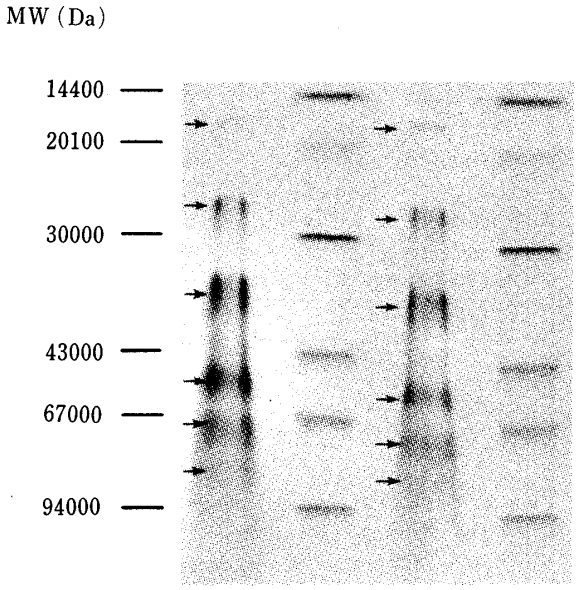


Fig. 6. SDS-PAGE of PEG-SOD Using a 10—15% Continuous Gradient Gel

Different modified proteins fractions are marked by arrows. Specific conditions are described in Materials and Methods.

TABLE I. Mobility of PEG-SOD Determined by SDS-PAGE

Protein	MW	log MW	*Rf* (mm)
α-Lactalbumin	14400	4.16	0.55
Trypsin inhibitor	20100	4.30	1.90
Carbonic anhydrase	30000	4.48	4.45
Ovalbumin	43000	4.63	7.60
Bovine serum albumin	67000	4.83	9.35
Phosphorylase b	94000	4.97	11.60
PEG-SOD band No. 1	—	—	1.25
PEG-SOD band No. 2	—	—	3.70
PEG-SOD band No. 3	—	—	6.05
PEG-SOD band No. 4	—	—	8.35
PEG-SOD band No. 5	—	—	9.65
PEG-SOD band No. 6	—	—	10.80

agreement with the reported MW of 31300.[15]

Changes in Chromatographic Properties

Reverse-Phase——Chromatograms in Fig. 7a show that native SOD elutes as a single sharp peak at 7.24 min while the PEG-modified SOD consists of at least two unequal sized peaks eluting at 10.21 and 12.10 min. (Fig. 7b). These results corroborate the previous findings from size-exclusion chromatography of multiple components in the modified enzyme. Some of the other major differences between the modified and native enzyme, as detected by this method, are the substantial increase in retention times and the broad peak shapes of the PEG modified SOD components, indicating an increase in species hydrophobicity and the heterogeneous nature of the resolved peaks.

Ion-Exchange——The modification of SOD that occurs by covalent attachment of PEG to accessible lysine and perhaps other nucleophilic amine residues results in many changes in the physicochemical characteristics of the enzyme. Since the modification involves the neutralization of positive charge (a substantial fraction of protein amines are protonated in the pH range 5—7), we would expect to see dramatic changes in the ion-exchange behavior of PEG-SOD, especially during cation exchange chromatography.

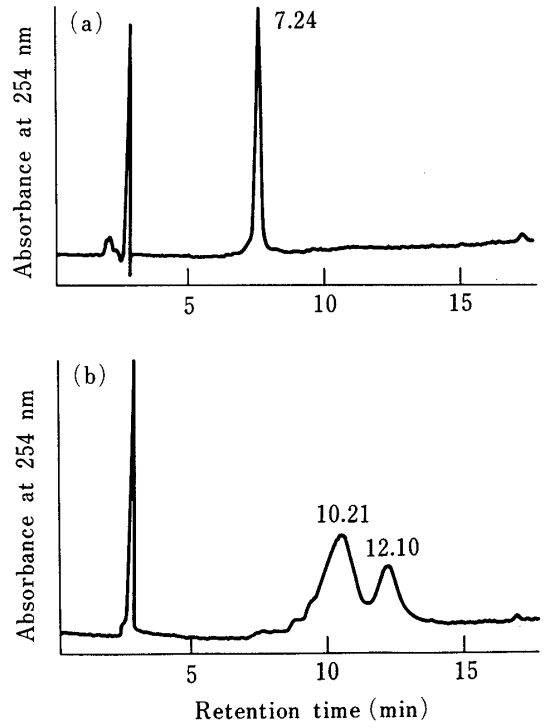


Fig. 7. Reverse-Phase Chromatograms Obtained Using a Vydac C_4 Column

Samples containing 100 μg SOD and 180 μg PEG-SOD were eluted using a gradient mobile phase described in Materials and Methods. (a) Native SOD. (b) PEG-SOD.

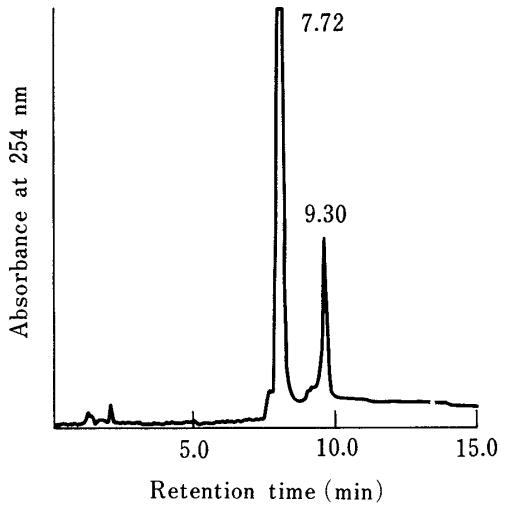


Fig. 8. Cation Exchange Chromatography of Unmodified SOD Using a Synchropak CM-300 Column and High Ionic Strength Gradient Elution

See Materials and Methods.

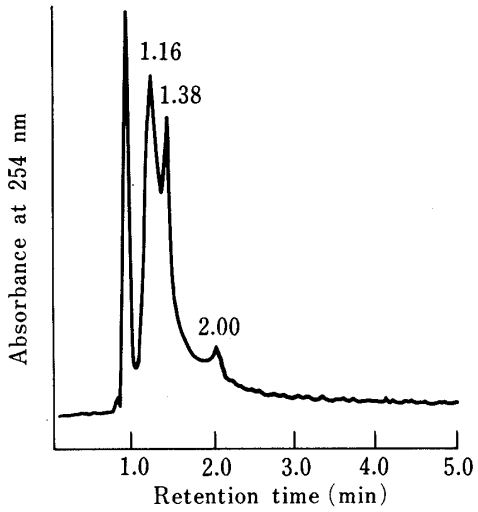


Fig. 9. Cation Exchange Chromatography of PEG-SOD Obtained by Low Ionic Strength Isocratic Elution

See Materials and Methods.

Chromatography on a weak cation exchange column at pH 5.0 resolves two peaks for the native protein, a major one at 7.72 min and a minor one at 9.30 min (Fig. 8). These results are consistent with others described in the text, which show SOD to be essentially homogeneous in nature. Chromatography of the PEG modified version of SOD using an identical chromatographic system resulted in a peak at the solvent front. In order to obtain any separation of the components of the modified enzyme mixture significant changes in the mobile phase were required. Under very low ionic strength isocratic conditions, separations as shown in Fig. 9 were obtained, with a solvent front peak at 0.84 min, two relatively large

peaks at 1.16 and 1.38 min and a small poorly resolved peak at 2.00 min. These results show that PEG-SOD consists of at least two major components and one minor species. The large change in chromatographic conditions required to effect a separation of PEG-SOD species *via* cation-exchange may best be explained on the basis of the number of positive charges on the PEG modified enzyme *vs.* the number on the native protein. In native SOD there are on the average 11.4 more amine groups present than in our particular batch of PEG-SOD, based on TNBS titrations of the two enzymes. Therefore the cation number (due to protonated amines) is significantly reduced upon modification, with perhaps a maximum of eight of the original twenty lysine residues present in SOD being able to interact in a cation exchange separation of PEG-SOD. A decrease in SOD isoelectric point of approximately one pH unit upon PEG modification (see below) also resulted in chromatography being performed at a pH above the pI of PEG-SOD. The substantial net decrease in electrostatic interaction, perhaps coupled with steric shielding, results in little retention of the PEG-modified species at pH 5.0, with elution near the solvent front even at very low ionic strength.

Anion exchange chromatography results in a sharp peak for native SOD at 8.16 min and a very broad peak for the modified enzyme at *ca.* 7.50 min (Figs. 10a and 10b). While the retention times of the two species are not markedly different, the elution pattern for PEG-SOD is much broader than for the native enzyme, which may again be indicative of multiple components. The small change in retention times can be rationalized by considering the type of interaction which occurs between the proteins and the bonded phase of the column. Under the chromatographic conditions employed (pH 7.5) a substantial and perhaps very similar fraction of both native and modified protein carboxyl groups will be ionized (the pI of SOD is 5.2). Since PEG modification occurs at free amine groups, the anion (carboxylate) number should not change significantly. Therefore, it is not unreasonable to expect a less dramatic difference in retention times for the two enzymes in an anion exchange system. Since there appear to be several components in PEG-SOD, the broad peak may be due to all components being very similar in anion number and retention, and essentially coeluting. Another possible reason for the broadness of the peak could be due to the polyethylene glycol chains attached to the enzyme, either interfering with the ion exchange process sterically[4,5,16] or effecting a mixed mechanism retention which could lead to band tailing.

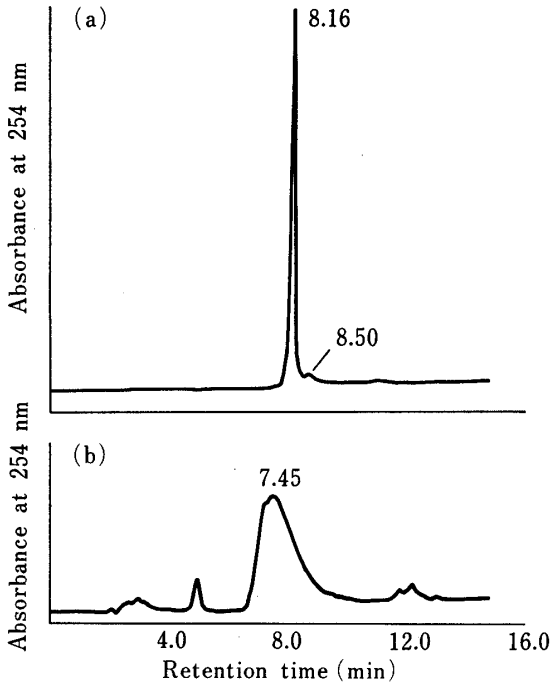


Fig. 10. Anion Exchange Chromatograms Obtained Using a Synchropak AX-300 Column

Samples containing 25 μg of SOD and 45 μg PEG-SOD were eluted using a sodium chloride gradient, as described in Materials and Methods. (a) Native SOD. (b) PEG-SOD.

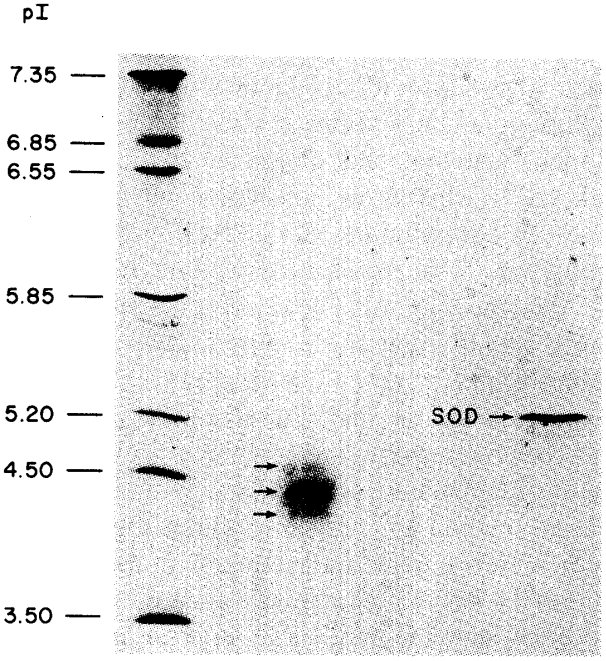


Fig. 11. Isoelectric Focusing of PEG-SOD on a pH 3.5 to 9.5 Gradient, Employing Conditions Described in Materials and Methods

Different PEG modified proteins species are marked by arrows.

TABLE II. Determination of pI of PEG-SOD

Protein	pI	Distance (mm)
Amyloglucosidase	3.50	88.9
Soybean trypsin inhibitor	4.55	71.3
β-Lactoglobulin	5.20	65.0
Bovine carbonic anhydrase	5.85	51.0
Human carbonic anhydrase	6.55	40.4
Horse myoglobin (acid band)	6.85	35.1
Horse myoglobin (basic band)	7.35	28.9
PEG-SOD band No. 1	—	
PEG-SOD band No. 2	—	76.1—69.9
PEG-SOD band No. 3	—	

Changes in Electrophoretic Properties

Isoelectric focusing of PEG-SOD resulted in the separation of at least three species, as shown in Fig. 11. Table II shows the results (equilibrium migration positions) of isoelectric focusing of PEG-SOD and marker protein standards on a gel with a pH gradient of 3.5 to 9.5. Isoelectric focusing on gels of narrower gradients resulted in dispersion of any band structure. Incomplete resolution of bands does not allow pI assignments for individual PEG-SOD species. However, the pI range can be estimated to be between 4.0 and 4.8 with a midpoint pI of 4.4. As a control, the pI of native SOD was determined in a manner similar to that employed for the PEG enzyme, and was found to possess an isoelectric point of 5.2 ± 0.2, in agreement with the established value.[17] A substantial decrease in pI upon PEG modification is consistent with the loss of an average of 11.4 protonated amines per SOD molecule (see above).

Discussion

Due to the nature of the chemical process used in the modification of primary amine groups in superoxide dismutase with polyethylene glycol[9] and the fact that twenty lysines of differing pK_a's and accessibilities are potentially available for modification, it is inevitable that the final product is a statistical mixture of PEG-SOD molecules with varying numbers of PEG chains attached. The extent of modification also depends upon the molar excess of activated PEG employed in the modification reaction.[9,18] A further contributing factor in the inhomogeneity of the preparation is the molecular weight range of the PEG-5000 used in the modification procedure, ranging from 4500 to 5500 Da.[19] These factors determine the overall distribution of PEG-SOD species and their properties in the commercial preparation. The unexpectedly high heterogeneity which can occur (as shown in this paper) will have serious implications in terms of the pharmaceutical utility of PEG-SOD and other similarly modified proteins used as therapeutic agents. In addition to perhaps being too polydisperse to be

acceptable as drug substances, the pharmaceutical analysis of PEG modified proteins will require special considerations.

High resolution analytical techniques which allow individual fractions of PEG-SOD species to be separated and measured, thereby allowing a characterization of the true natures of various PEG-SOD preparation, can be developed. Knowing that any given batch of PEG-SOD will be polydisperse, we compared the ability of various bioanalytical techniques in terms of their ability to resolve different PEG-SOD components. SDS-PAGE is the most sensitive of the techniques examined for separating the various molecular weight fractions of PEG-SOD. With this method six species were detected (see Fig. 6). The relative distribution of species is neither uniform nor a simple gaussian, instead suggeting a somewhat asymptotic decrease in species population with increasing molecular weight. The degree to which the extent of PEG modification affects the Coomassie staining and therefore the apparent amounts of various molecular weight PEG-SOD species is unknown. Electrophoretic results can therefore (except for mobilities) be assessed in only a qualitative fashion. The molecular weight range detected extends from approximately the weight of the native enzyme to a molecular weight which is actually higher than would be predicted theoretically on the basis of modification of all twenty available lysine residues (see below). The lowest molecular weight fraction detected corresponds to native unmodified enzyme while the highest molecular fractions could be conventionally explained as perhaps being due to crosslinking of SOD molecules with bifunctional activated PEG or to modification of nucleophilic side chain residues other than lysine.

The effect of covalently attached hydrophilic random coil polymers on the migration of an SDS saturated denatured protein through an acrylamide gel matrix may also be substantial. Non-SDS-denatured electrophoretic mobility of proteins other than SOD decreases significantly upon attachment of PEG, the decrease being much greater than would be expected from the increased size of the adduct alone. For example, bovine serum albumin modified with either 1900 or 5000 Da PEG was found to move very slowly during nongradient acrylamide gel electrophoresis.[4] It appeared that the PEG-albumin became immobilized in the first 1—2 mm of a 4% gel with 2% crosslinking. Other studies performed on PEG-modified enzymes reveal a decrease in migration as the degree of modification increases. The question of whether the decreased mobilities of the PEG-modified enzymes were caused by PEG entanglement in the acrylamide gel was examined by thin layer electrophoresis.[20] Preparations of PEG-phenylalanine ammonia lyase (having varying percentages of PEG attachment) were electrophoretically resolved using Sephadex G-75 as the anticonvection medium, where entanglement of polyethylene glycol chains in a polyacrylamide matrix could not occur. Since the migration distances in this system were directly related to the percentage of PEG-modification, it was concluded that entanglement of PEG chains is not the only mechanism leading to anomalously low mobility, but that in addition there exists a charge shielding effect of the hydrophilic PEG-shell surrounding the protein. Addition of free PEG to gels employed in SDS-PAGE of conventional proteins has also resulted in decreased protein mobility.[21]

Isoelectric focusing of PEG-SOD resolved three broad bands with pI's varying from 4.0 to 4.8 (see Fig. 11). As would be expected on the basis of neutralization of positive charge upon PEG attachment to lysines, all pI's are lower than that of the native protein (pI = 5.2). Substantial microheterogeneity appeared to exist, in that resolved bands remained broad, regardless of experimental conditions and pH gradients employed.

Although chromatographic analysis of PEG-SOD results in a separation of components in PEG-SOD, such techniques are not as selective as the electrophoretic methods examined. Size-exclusion chromatography (Fig. 4) partially separates the modified protein preparation into two broad and very poorly resolved bands. Similarly broad peaks have been observed

during size-exclusion analysis of other preparations of PEG-SOD.[7,9] Reverse-phase chromatography also resolved PEG-SOD into only two broad bands (Fig. 7b).

Anion-exchange chromatography revealed only one very broad band for PEG-SOD chromatographed under the same conditions as the native enzyme (Fig. 10b). The broadness of peaks observed with all the abovementioned chromatographic techniques indicates incomplete resolution of what from SDS-PAGE are known to be at least six species.

Previous anion-exchange chromatographic analyses of PEG-albumins showed all fractions eluting in the void volume.[4] Since unmodified serum albumin and SOD have similar pI's (4.9 *vs.* 5.2), the marked difference between our results and those obtained by others for PEG-albumin suggests marked variability in either the extent of PEG modification, or the effect of modification, between different proteins.

Cation-exchange chromatography of PEG-SOD (Fig. 9) resulted in three moderately well resolved peaks. In order to achieve any retention, a very low ionic strength isocratic mobile phase was required when performing chromatography at a pH identical to that employed during the analysis of native SOD, where we employed a much higher ionic strength mobile phase in a gradient system. These chromatographic analyses demonstrate the extremely large difference in surface positive charge between the native and PEG-modified enzymes and may be in part responsible for the altered biological properties of the latter species.

Molecular weights of proteins are typically determined by measurement of their mobility or diffusivity through a defined medium. Attachment of multiple chains of uncharged hydrophilic, random coil polyethylene glycol to a protein (in either native globular or denature random coil conformations), may significantly affect a protein's hydrodynamic radius, and therefore measurements based on this value such as molecular weights. The effect of PEG modification on the apparent average molecular weight of a protein was determined (in the case of SOD) by comparing molecular weights derived from SDS-PAGE, size exclusion chromatography, and photon correlation spectroscopy, with the true average molecular weight obtained by a titration which determines the number of amino groups which have been modified with PEG of a known molecular weight.

Titration of remaining free amines with TNBS showed that an average of 11.4 lysines had been modified with PEG 5000 in the batch of PEG-SOD examined, giving a nominal molecular mass of 86000 Da. A comparison with molecular weights obtained for partially resolved mixtures of PEG-SOD species, by the abovementioned methods, is shown in Table III. While it is difficult to compare the average molecular weight obtained by titration with other values representative of partially resolved fractions of the PEG-SOD mixture, examination of Table III shows that size-exclusion chromatography gives values which are consistently lower than the true mean, and electrophoresis provides a molecular weight estimate significantly larger than any of the other measured values.

All the analytical separation techniques employed in this study showed that in the case of

Table III. Comparison of PEG-SOD Molecular Weights Obtained by Various Techniques

Method	MW (Da)
TNBS titration	86000
Light scattering[a]	*ca.* 95000[b]
Size-exclusion chromatography	< 50000—*ca.* 75000
SDS-PAGE	34000—162500

a) Photon correlation spectroscopy. *b*) Based on diameter of equivalent diameter random coil polymer.

the product batch examined, PEG modification results in substantial product heterogeneity. At least six species were separated by SDS-PAGE, with this and all other techniques yielding broad bands and peaks indicating incomplete resolution, and additional microheterogeneity within fractions. A product mixture of this type (but not necessarily of this high dispersity) is to be expected since the probability of modification of a given lysine residue is statistical in nature, and dependent upon individual pK_a's and steric accessibilities. The molar excess of activated PEG employed while determining the net degree of modification[7,18,22] may perhaps also affect product dispersity. Heterogeneity of PEG-enzymes may also depend on the identity of activated PEG starting material,[10] and on the specific properties of the protein being modified.[23] Careful control of reaction conditions to minimize dispersity and maximize reproducibility of species distributions will therefore be required for any PEG-protein employed as a therapeutic agent. Careful analysis to assure batch to batch consistency and acceptability will also be required.

PEG modification of proteins can result in substantial changes in their properties. In the case of SOD, commercial modification using hydroxy succinimide activated polyethylene glycol resulted in the formation of at least six species, with isoelectric points 0.4 to 1.2 pH units lower than the native enzyme. The modified protein is also significantly more hydrophobic, as measured by reverse-phase chromatography, even though PEG 5000 is a highly water soluble species. Diffusivity through water, as measured by photon correlation spectroscopy, is reduced by PEG modification to an extent that PEG-SOD behaves like a random coil polymer of *ca.* 95000 Da, suggesting that the "hybrid" globular protein with attached random coils behaves similarly to a random coil polyethylene oxide molecule of equivalent molecular weight. Anomalously large species (apparent MW) are observed by SDS-PAGE, possibly indicating entanglement of PEG chain in the polyacrylamide matrix resulting in a decrease in apparent mobility (and apparent increase in molecular weight of PEG-SOD). At pH 5.0 the net accessible cationic charge of PEG-SOD is very small, as evidenced by the almost complete lack of retention during cation exchange chromatography.

There are several potential biological implications to the above results. It is unclear what the enzymatic activities of individual fractions are. In the case of SOD, it has been shown that several of the PEG modified fractions can be completely inactive.[10] The mechanism of inactivation is unknown. Damage to the enzyme active site appears unlikely, since the spectrum of the catalytic site copper remained unchanged in the batch of PEG-SOD examined. Possible conformational changes induced by PEG attachment also do not appear to be the primary cause of activity loss since the native enzyme remains active even after subunit dissociation under structurally perturbing conditions.[24] Since catalytic activity of SOD is due in part to the existence of a positive electric field which increases the association rate with superoxide anion by at least a factor of thirty,[25] it is possible that the decrease in number of ionizable lysines upon PEG-modification decreases activity to the observed value. The marked change in electrostatic binding properties and moderate change in hydrophobicity and isoelectric point, may also be in part responsible for the observed major decrease in immunogenicity and increase in plasma half life, usually attributed to steric shielding of the polypeptide backbone in PEG modified proteins.

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