Characterization of the heterogeneity of polyethylene glycol-modified superoxide dismutase by chromatographic and electrophoretic techniques

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ABSTRACT

Covalent attachment of polyethylene glycol (PEG) chains to the enzyme Cu,Zn-superoxide dismutase (SOD) produces a heterogeneous mixture of modified protein species. The heterogeneity of the product (PEG–SOD) derives from a variable stoichiometric combination of PEG with individual SOD molecules in addition to the polydispersity of the PEG reagent. Characterization of PEG–SOD presents significant challenges due in part to this heterogeneity in addition to the hybrid nature of the modified enzyme. The application of classical methods of protein characterization is not always successful for these PEG–proteins requiring the development of alternative or modified procedures. A series of chromatographic techniques including reversed-phase, ion-exchange, size-exclusion, and hydrophobic interaction high-performance liquid chromatography along with electrophoretic techniques including isoelectric focusing, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and capillary zone electrophoresis have been developed for assessing the degree of heterogeneity of PEG–SOD samples which encompass a range of different stoichiometries. Examples will be given demonstrating the application of these techniques to characterize PEG–SOD samples of different composition produced during the course of the reaction between SOD and an activated PEG reagent.

INTRODUCTION

With the emergence of biotechnology there has been a substantial increase in the investigation of proteinaceous materials as therapeutic agents. While these materials typically possess high specificity and activity, formulating and delivering such pharmaceuticals presents significant problems. An additional problem is the potential immunogenicity of these products, which is especially relevant to the use of exogenous proteins. Covalent attachment of polyethylene glycol (PEG) chains to potential therapeutic proteins has shown promise for increasing *in vitro* stability, *in vivo* half-life, and protein solubility as well as decreasing immunogenicity [1,2]. Several examples exist demonstrating the advantages of this process for improving one or more of these properties for proteins including Cu,Zn-superoxide dismutase (SOD) [3], catalase [4], adenosine deaminase (ADA) [5], asparaginase [6], inter-leukin-2 (IL-2) [7] and streptokinase [8], among others.

The process of attaching PEG to the protein is typically achieved by making one of several different types of activated PEG reagents [1,2,9,10] react with accessible primary amine (primarily lysine) residues on the protein, although other amino acid residues have also been targeted [11]. Since a typical protein will possess a number of such groups, each having different reactivities and degrees of accessibility, the resulting product contains a family of species which is characterized by a distribution in both the number and position of attachment of the PEG groups. Another factor complicating this situation is the inherent polydispersity which is typical of a polymer such as PEG (nominal molecular weight of 5000 for this study).

Since the properties of the PEG–protein product may depend on its composition, it is important to be able to characterize this heterogeneity in order to

assure product consistency. For SOD, which has 20 possible PEG attachment sites per dimer, the theoretical maximum number of species produced on reaction with PEG is approximately 20^{20}. This theoretical maximum does not include the polydispersity of the PEG reagent which will be superimposed over the site attachment heterogeneity. The actual number of different PEG–SOD species will be less than the theoretical maximum since not all lysines will typically be derivatized and reaction conditions can be optimized to exercise some control over the product composition. Still, it is clear that the product will be quite heterogeneous and it is not practical to try to separate all possible species. Instead, the goal is to establish a separation pattern which can serve as a type of fingerprint which can be used to distinguish between samples of different composition and degrees of heterogeneity.

The characterization of PEG–proteins has been addressed by several investigators with varying degrees of success [12–14]. In most cases this involved the application of classical protein characterization methods which generally did not have the necessary degree of resolution to address this heterogeneity issue. McGoff et al. [13] provided a fairly comprehensive evaluation of PEG–SOD using standard protein methods. Except for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and to a lesser extent isoelectric focusing (IEF), the authors found these classical techniques to be inadequate in characterizing product heterogeneity. Recently, Cunico et al. [15] presented a new procedure involving charge-reversal capillary zone electrophoresis (CZE) for separating components of some model PEG-proteins. Depending on the length of the PEG chain, these authors were able to separate up to four components. Emerging technologies such as high-molecular-weight mass spectrometry using an electrospray interface have also shown some promise for characterizing PEG–proteins [16].

For the current study, the reaction of SOD with an activated succinyl succinate ester of PEG (see Fig. 1) was evaluated at different time points during the course of the reaction using chromatographic and electrophoretic techniques. Emphasis was placed on the ability of these methods to characterize the distribution of modified species in the product at the different stages of the reaction. In several
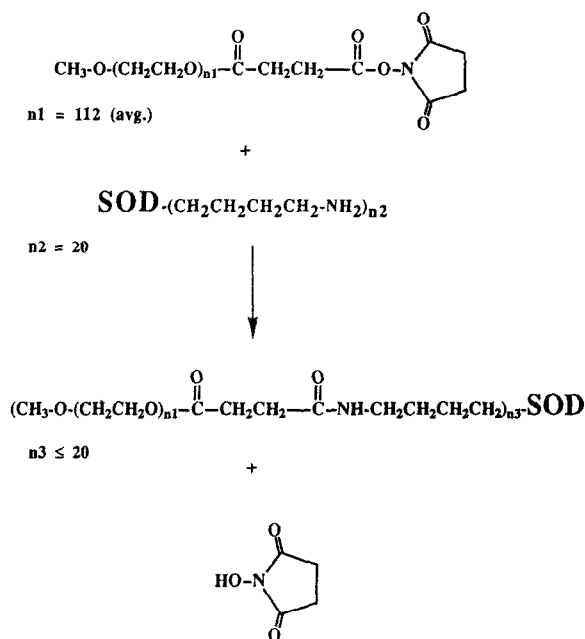

Fig. 1. Reaction used to prepare PEG–SOD.

cases, where classical methods were found to be inadequate, new approaches or modified procedures were developed enabling the separation of several fractions representing different degrees of PEG modification. The identification of the specific lysine residues on SOD which reacted with the activated PEG reagent is beyond the scope of this paper, although others have addressed the site attachment distribution as a function of the total number of modified lysine residues [16,17].

EXPERIMENTAL

Chemicals

Bovine erythrocyte SOD was from DDI Pharmaceuticals (Mountain View, CA, USA). PEG–SOD and the succinyl succinate ester of PEG (PEG-SS) were prepared in our laboratory. Glycine and ammonium sulfate were obtained from Bio-Rad (Richmond, CA, USA). Acrylamide, N,N-methylenebisacrylamide, Coomassie blue G-250, ammonium persulfate, molecular weight and isoelectric focusing markers and Ampholine mixtures pH 4.0–6.5 and 2.5–4.0 were purchased from Pharmacia LKB (Piscataway, NJ, USA). Histidine hydrochloride

was obtained from Fluka Biochemika (Buchs, Switzerland). Urea and SDS were purchased from J. T. Baker (Phillipsburg, NJ, USA). Fluorescamine was obtained from Pierce (Rockford, IL, USA). All other reagents, solvents and buffers were of the highest quality available.

Preparation of PEG-SOD

The temperature for the synthesis of PEG–SOD was held constant at 30°C using a Multitemp II thermostatic circulator (Pharmacia LKB). To 100 ml of a 5.1-mg/ml solution of SOD (0.016 mmol) in a pH 7.80, 0.1 F sodium phosphate buffer, was added 1.70 g (0.327 mmol) of PEG-SS while stirring at high speed to affect rapid dissolution. The stir rate was reduced and the reaction pH was held constant at pH 7.80 using a Mettler Model DL25 titrator operated in the pH-stat mode by the addition of 0.5 F NaOH.

Chromatographic equipment

For the determination of the average extent of modification of SOD, a high-performance liquid chromatographic (HPLC) system consisting of a Waters (Waters, Millipore, Milford, MA, USA) WISP Model 712 autoinjector, a Waters Model 600E system controller and pump and an ABI (Foster City, CA, USA) Model 980 fluorescence detector was used. The high-performance size-exclusion chromatographic (HPSEC) system for the evaluation of the heterogeneity of PEG-SOD consisted of a Waters WISP Model 712 autoinjector, a Waters Model 590 pump, and an ABI Model 757 detector. Chromatographic data obtained from the Waters HPLC systems were collected and processed by the PENelson Access*Chrom Model 6000 data system (Cupertino, CA, USA) on a VAX computer. High-performance hydrophobic interaction chromatographic (HPHIC) reversed-phase (RP) HPLC and high-performance ion-exchange chromatographic (HPIEC) analyses were performed using a Hewlett-Packard (Avondale, PA, USA) 1090 diode-array HPLC system equipped with a HP chromatography data station.

Determination of the extent of modification of SOD

The average degree of modification of SOD was evaluated by determining the number of unreacted lysines remaining on SOD using fluorescamine de-

rivatization. At appropriate time intervals during the synthesis of PEG–SOD, 100-μl aliquots of the reaction mixture were removed and immediately quenched with excess glycine (5.00 ml of a 0.235-mg/ml glycine solution). The fluorescamine derivative was prepared by mixing 250 μl of the quenched sample with 250 μl of a pH 9.0, 0.2 F H_3BO_3 buffer. Then, while vortexing this mixture, 500 μl of a 3.0 mg/ml fluorescamine solution was added in a drop-wise fashion. Separation of the SOD/PEG–SOD fluorescamine derivatives from lower-molecular-weight interfering substances (*i.e.* < 1000 dalton, including glycine) was performed on a 300 × 7.8 mm Bio-Gel 20 XL chromatographic column (Bio-Rad) using a mobile phase consisting of acetonitrile–0.15 F H_3BO_3, 0.2 F NaCl buffer pH 9.0 (20:80, v/v), containing 0.28% (w/v) SDS. All peaks were eluted from the column at a flow-rate of 1.0 ml/min and detection was accomplished using a fluorescence detector with an excitation wavelength at 390 nm and a 480 nm emission cut-off filter. A 20-μl injection volume was used. Further details of this method may be found elsewhere [18].

Additional quantities of the reaction mixture were removed at the 0, 1, 4 and 50 min time points for evaluation by chromatographic and electrophoretic methods. For each of these samples the reaction was quenched in excess glycine as stated above and the resulting PEG–SOD product concentrated to approximately 2.5 mg/ml using a Centriprep ultrafiltration tube having a 10 000 molecular weight cut-off filter (Amicon, W. R. Grace & Co., Beverly, MA, USA) to remove some of the lower-molecular-weight substances.

HPSEC. Separations were performed on a 300 × 7.8 mm Progel-TSK G3000SWXL column (Supelco, Bellefonte, PA, USA) using a mobile phase consisting of methanol–pH 6.8, 0.1 F NaH$_2$PO$_4$, 0.2 F NaCl buffer (45:55, v/v). A 1.0-ml/min flow-rate, a 20-μl injection volume, and UV detection at 214 nm were used.

HPIEC. Cation-exchange separations were performed on a 35 × 4.6 mm TosoHaas SP-NPR column using a gradient mobile phase consisting of (A) pH 2.6, 0.02 F sodium phosphate; (B) pH 2.6, 0.02 F sodium phosphate, 1 F NaCl. The gradient was varied linearly from 0–100% B over 15 min at a flow-rate of 1.0 ml/min. The column was cooled to 10°C using the thermostatic circulator. An injection vol-

ume of 20 μl and UV detection at 220 nm were used.

RP-HPLC. Reversed-phase separations were performed on a 35 × 4.6 mm TosoHaas C18-NPR column using a gradient mobile phase consisting of (A) pH 7.0, 0.02 F sodium phosphate; (B) isopropanol. The gradient was varied as specified in the appropriate figures. A 1.0-ml/min. flow-rate, a 10-μl injection volume (5 μg), and UV detection at 214 nm were used.

HPHIC. Hydrophobic interaction separations were performed on a 35 × 4.6 mm TosoHaas butyl-NPR column using a gradient mobile phase consisting of (A) pH 7.0, 2 F ammonium sulfate, 0.1 F sodium phosphate; (B) pH 7.0, 0.1 F sodium phosphate. The gradient was varied linearly from 10–80% B over 40 min at a flow-rate of 1.0 ml/min. An injection volume of 10 μl (5 μg), and UV detection at 214 nm were used.

SDS-PAGE. Separations were performed on a Pharmacia Phast System using a precast 0.45 mm PhastGel, gradient 8–25, at a uniform temperature of 15°C. The system was programmed to apply a maximum of 3.0 W, 250 V, and 10 mA for a 70-volthour time period. Gels were stained in Coomassie Blue G-250 stain in the leuco form [19] overnight and were then washed in water for 4 h.

IEF. Analyses were performed on a horizontal LKB Multiphor II electrophoresis unit connected to a LKB Multitemp II thermostatic circulator and a Macrodrive 5000 power supply. Separations were performed using an in-house prepared 188 × 115 × 1 mm, 4% T, 6% C polyacrylamide gel[a] containing 9 M urea and 2.0% pH 4.0–6.5 Ampholine. The anode wick was soaked in 2% pH 2.5–4.0 Ampholine and the cathode wick was soaked in pH 7.0, 0.2 F histidine. The electrophoretic system was programmed to apply a maximum of 8 W, 1000 V and 15 mA for 90 min which was then increased to 16 W, 1420 V and 15 mA for an additional 90 min. The temperature was uniformly maintained at 17°C. The resulting gel was stained in Coomassie blue G-250 stain in the leuco form as stated above.

CZE. Two systems were used. System A was a Beckman PACE 2000. System B was a laboratory-built modular system consisting of a Bertan Model 230 30-kV power supply, platinum wire electrodes, and a Linear Model 204 UV detector (Reno, NV,

USA). Fused-silica capillary tubing for all work (50 μm I.D. × 357 μm O.D.) was from Polymicro Technologies (Phoenix, AZ, USA). Capillaries were washed with 1 F NaOH for 30 min, water for 5 min and equilibrated with operating buffer for 20 min. Data for all experiments was collected using PE-Nelson Access★Chrom software at a sampling rate of 3 Hz.

RESULTS AND DISCUSSION

The extent of derivatization of the lysines on SOD during the course of the reaction with the activated ester of PEG was determined using the method described in the Experimental section. Further details of this method can be found elsewhere [18]. The graph in Fig. 2 depicts the time course of the reaction where the y-axis represents the percentage of lysines on SOD (20 per SOD dimer) derivatized with PEG.

HPSEC

Since each PEG chain that is attached to SOD will contribute on average 5000 dalton to the total mass of the modified protein (16% of the native protein weight), HPSEC is a natural choice for trying to separate different species. Fig. 3 shows a series of HPSEC chromatograms of the four different samples of modified SOD (0, 5, 19, and 37% mod-
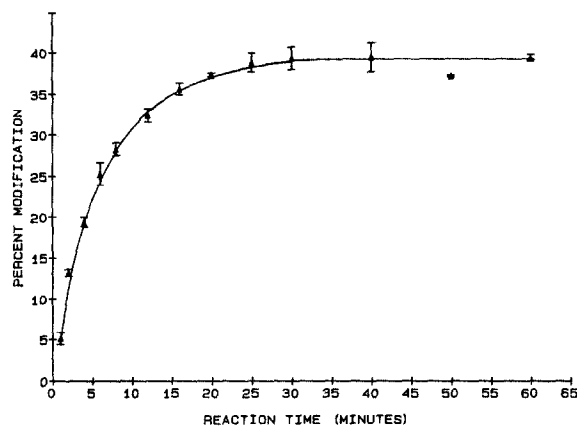

Fig. 2. Curve depicting the cumulative percent modification of lysines on SOD during the course of the reaction used to prepare PEG–SOD.

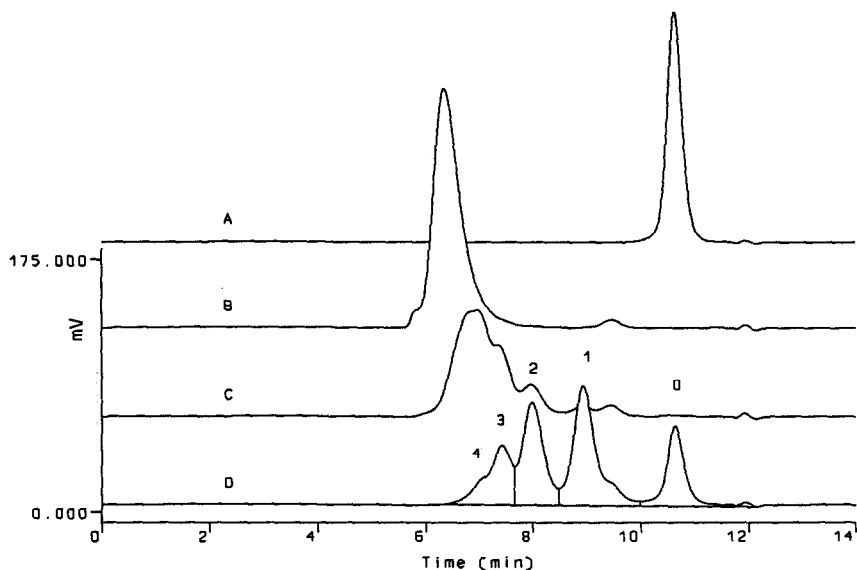[a] T = [g acrylamide + g N,N′-methylenebisacrylamide (Bis)]/100 ml solution; C = g Bis/% T.

Fig. 3. HPSEC chromatograms of (A) unmodified SOD, (B) 50-min PEG–SOD sample, (C) 4-min PEG–SOD sample and (D) 1-min PEG–SOD sample. Numbers over the peaks refer to the predicted number of PEG chains per SOD dimer.

ified). The unmodified SOD elutes at 10.7 min. The peak at 9.5 min is due to residual PEG reagent. The next peak at about 9 min is assigned to a mono-PEG–SOD species followed at successively shorter elution times by the di-, tri- and tetraPEG–SOD species. The exact identity of these peaks has not been independently confirmed but is based on their relative positions in the chromatogram in addition to the known average degree of modification of the protein determined using the method described in the experimental section. After four PEGs per SOD dimer the resolution drops off dramatically. This fact is not unexpected based on the hybrid nature of the modified enzyme (globular protein core with a shell of random coil polymers). With the addition of successive PEG chains to the protein, the relative difference in molecular size between individual species drops off considerably correlating to lower chromatographic resolution.

HPIEC

In addition to a change in the mass of the protein on addition of PEG chains to SOD, there is also a change in total protein charge due to the conversion of the effected lysine residues to amides. Therefore, it would be expected that HPIEC should be able to discriminate among the different PEG–SOD spe-

cies. In fact, we have found, as have others [10,13,14], that HPIEC of PEG–proteins is difficult. This is apparently due to the PEG chains sterically interfering with the interaction of charged residues on the protein with the ion-exchange support or a shielding of protein charge by PEG [13].

A further complicating factor, which is generic to all modes of HPLC analysis of PEG–proteins, is their significant hydrodynamic size. Due to the random coil nature of PEG compared to the globular folded structure of the protein, the effective hydrodynamic size of PEG–SOD (in relation to protein standards) is found to be several times larger than the native protein (data not shown). The larger size of PEG–SOD results in slower diffusion kinetics through traditional porous supports correlating into poorer resolution. To circumvent these problems, a different strategy for analyzing PEG–SOD was used. This involved the use of a non-porous support in combination with a low pH eluent. Non-porous supports provide for better mass transfer kinetics for large molecules [20]. The low pH eluent maximizes the net positive charge on the modified protein enabling retention on a cation exchanger.

Fig. 4 shows a series of HPIEC chromatograms obtained on the different PEG–SOD samples. The complexity of these chromatograms is in stark con-
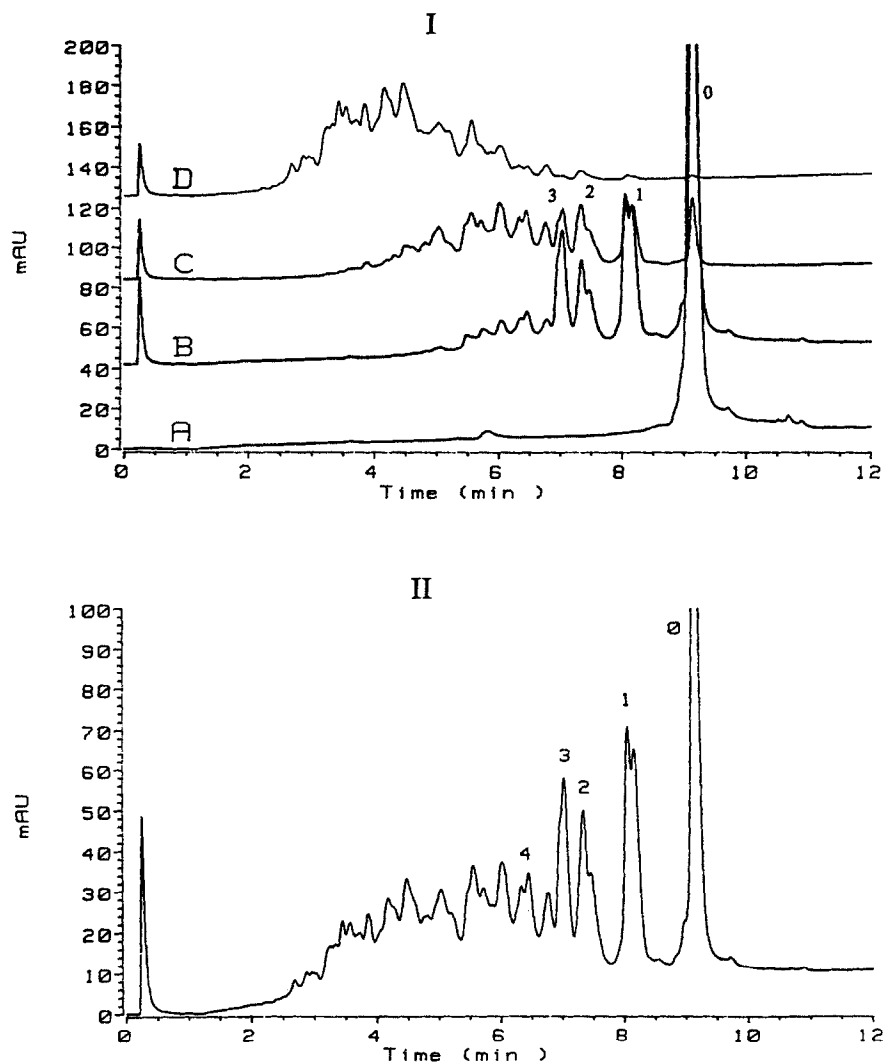
Fig. 4. HPIEC chromatograms of PEG–SOD samples. (I): Chromatograms of (A) unmodified SOD, (B) 1-min PEG–SOD sample, (C) 4-min PEG–SOD sample and (D) 50-min PEG–SOD sample. (II): HPIEC chromatograms of a mixture of the samples in (I). Numbers over peaks refer to the predicted number of PEG chains per SOD dimer.

trast to those obtained by HPSEC and they begin to depict the heterogeneity of the samples. A series of experiments was run to demonstrate that these peaks represent real components of the sample rather than artifacts produced during the chromatographic process. Close examination of these chromatograms reveals a pattern in which peaks can be grouped into subsets which are interpreted to represent mono-, di-, tri-, etc. PEG–SOD species. The multiple bands within each subset would then represent the isomers differing in the sites of attachment and/or length of the PEG chains. The chromatographic profiles of these samples reflect the trend that would be expected based on the known average degree of modification of the samples. That is, the higher the degree of modification, the shorter the retention time and the greater the number of possible species. This leads to more complex chromatograms with decreasing definition between the major fractions.
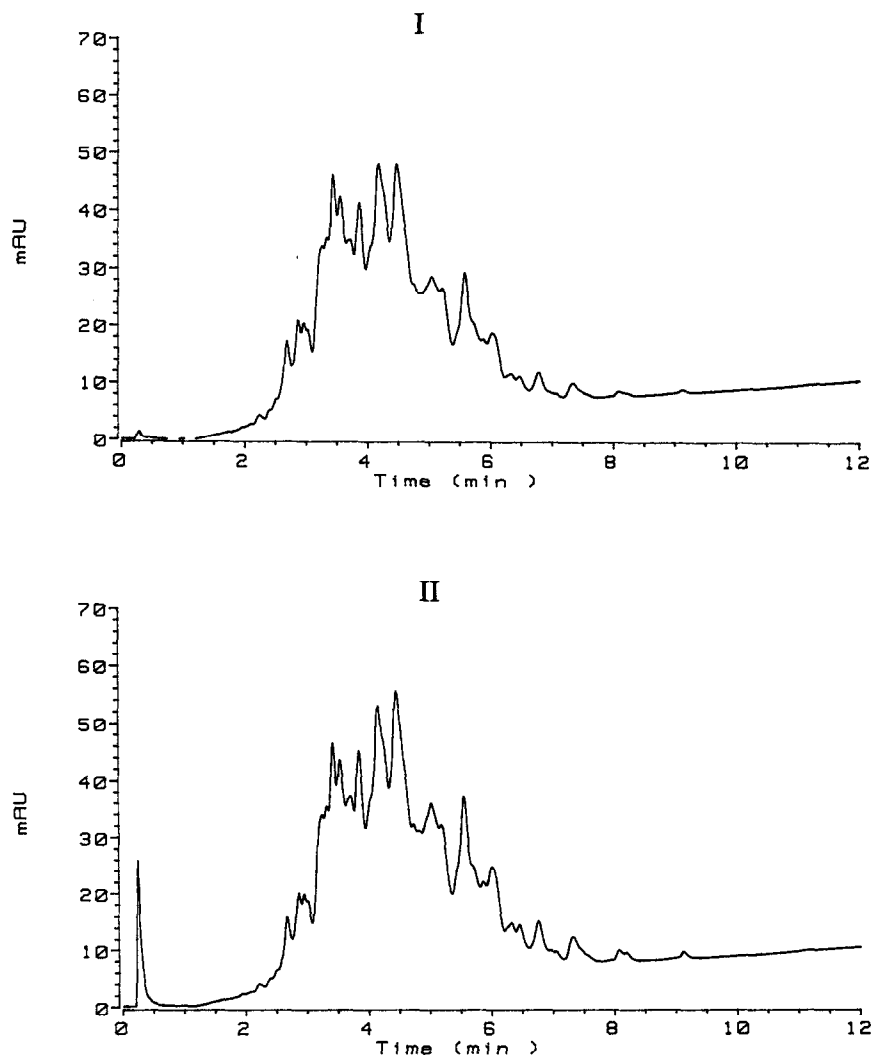
Fig. 5. HPIEC chromatograms of two different samples of PEG–SOD; (I) production scale sample, (II) small lab scale sample.

The potential utility of this technique is demonstrated in Fig. 5 which shows a comparison of two chromatograms of different samples of PEG–SOD prepared using the same reaction conditions but at different times and on different scales (small lab scale *versus* a larger production scale). The peak patterns are identical, indicating that the chromatograms can serve as a type of fingerprint to establish product consistency from lot to lot. It should be noted that chromatography of these same samples on a traditional large-pore support of the same chemistry resulted in very little resolution of individual peaks as shown in Fig. 6, clearly demonstrating the advantages of non-porous supports for PEG-proteins.

RP–HPLC

Although PEG is generally considered a hydrophilic polymer, under RP-HPLC conditions the effect of the addition of PEG to a protein is to increase its hydrophobicity and thus, its retention time relative to the unmodified protein. As was the case for HPIEC, a new approach was used to analyze PEG–SOD by RP-HPLC. This entailed the use
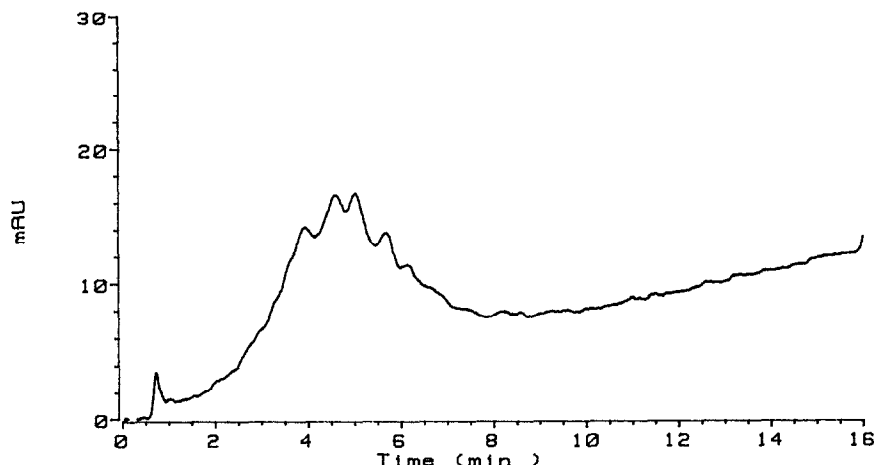
Fig. 6. HPIEC chromatogram of 50-min PEG–SOD reaction product obtained on a traditional porous support. Chromatography was performed using an Ultropak 75 × 7.5 mm TSK SP-5PW (LKB Pharmacia) at a flow-rate of 3.0 ml/min at 10°C with a 15-min linear gradient from 0–100% B; mobile phase A = pH 2.6, 0.02 F sodium phosphate; mobile phase B = pH 2.6, 0.02 F sodium phosphate, 1 F sodium chloride. The injection volume was 20 μl and detection was accomplished at 220 nm.

of a non-porous RP-HPLC support in conjunction with a non-denaturing mobile phase at pH 7. Fig. 7 contains chromatograms of the different PEG–SOD samples obtained using an isopropanol gradient at pH 7.0. These conditions were optimized so that unmodified SOD was not retained and, therefore, retention is due solely to the attached PEG chains. Under these conditions, the first group of peaks that elutes is expected to be the monoPEG–SOD species followed in order by the di-, tri-, etc. species.

As was the case with HPIEC, the chromatograms appear to be composed of a series of major peaks each of which displays some fine structure. The interpretation of these chromatograms is the same as for the corresponding HPIEC data.

A further insight into the high degree of heterogeneity possible with PEG–SOD is shown in Fig. 8. This figure represents chromatograms of the same two samples of PEG-SOD as depicted in Fig. 5. These RP-HPLC chromatograms were obtained using the same conditions used in Fig. 7 but with a shallower gradient profile. Again, the profiles of these samples were found to be nearly identical.

HPHIC

Since HPHIC relies on the same type of forces (partitioning into a hydrophobic phase) encoun-

tered in RP-HPLC, except in a diminished capacity, it would be expected that the two methods would produce similar results. As was the case for HPIEC and RP-HPLC, a non-porous support was used along with a traditional inverse ammonium sulfate gradient. The chromatograms in Fig. 9 show peak profiles for these samples that are very similar to the corresponding RP-HPLC chromatograms. A notable difference is the absence of fine structure in the major peak fractions which results in a clearer discrimination between adjacent peaks in the HPHIC chromatograms. A speculative explanation for this difference between the two techniques is that HPHIC responds more to the bulk properties of the molecule such as solubility which are determined more by the total number of attached PEG chains and less by the positioning of these chains along the polypeptide chain. RP-HPLC, on the other hand, is more of a surface interaction technique. Therefore, the positioning and size of the PEG chains on SOD exert more influence on the separation process resulting in more separation of these isoforms.

SDS-PAGE

The results of SDS-PAGE analysis of the PEG–SOD samples are shown in Fig. 10. Samples were heated with SDS and 2-mercaptoethanol to break the SOD dimer into its subunits and reduce the di-
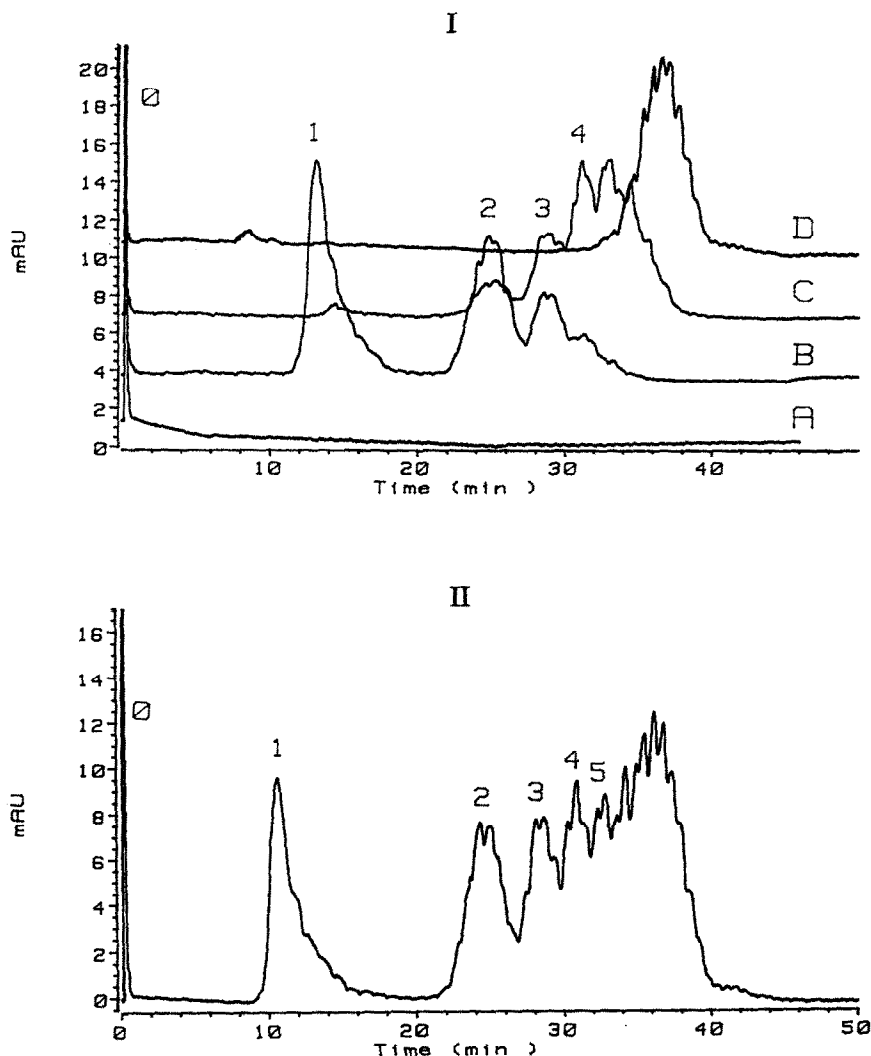
Fig. 7. RP-HPLC chromatograms of different samples of PEG–SOD using the following gradient profile: 5–7% B in 5 min and then 7–16% B in 45 min. Refer to the Experimental section for the remaining chromatographic conditions. (I): Chromatograms of (A) unmodified SOD, (B) 1-min PEG–SOD sample, (C) 4-min PEG–SOD sample and (D) 50-min PEG–SOD sample. (II): Chromatogram of a mixture of the samples in (I). Numbers over the peaks refer to the predicted number of PEG chains per SOD dimer.

sulfide linkages. Because of the high solubility of PEG–proteins even after fixing with trichloroacetic acid, detection of the separated protein bands was problematic due to loss of sample during the washing step. Therefore, Coomassie blue G-250 stain prepared in the leuco form was used so that the fixing and staining of the protein could be performed in a single step. Band assignments for the samples are given in the figure. The separation of bands is quite good up to four PEGs per SOD monomer, although the calculated molecular weights based on the protein standard calibration are not in good agreement with theory. This may be due to the difference between the binding of SDS to PEG (on the PEG–SOD) and SOD which is explained further under the CZE section below. Another problem with this technique is that it is unknown how the PEG will effect the staining procedure and, there-
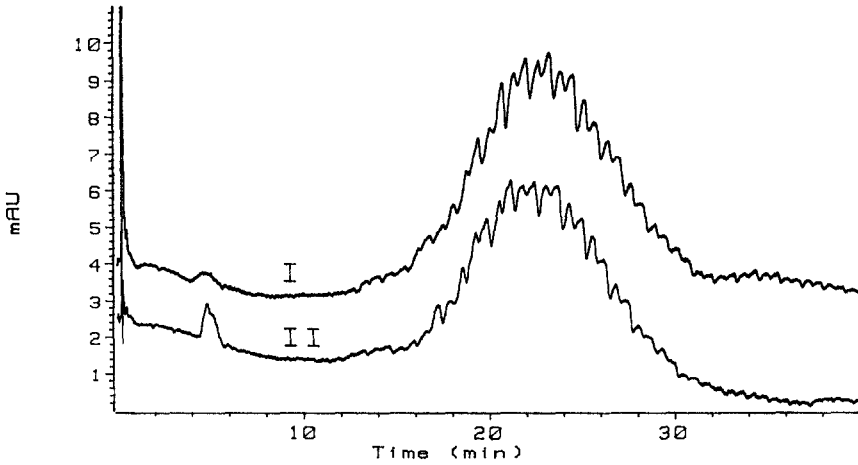
Fig. 8. RP-HPLC chromatograms of the two samples of PEG–SOD depicted in Fig. 5. Chromatograms obtained using the following gradient profile: 5–11.5% B in 5 min and then 11.5–14.9% in B in 40 min. Refer to the Experimental section for the remaining chromatographic conditions.
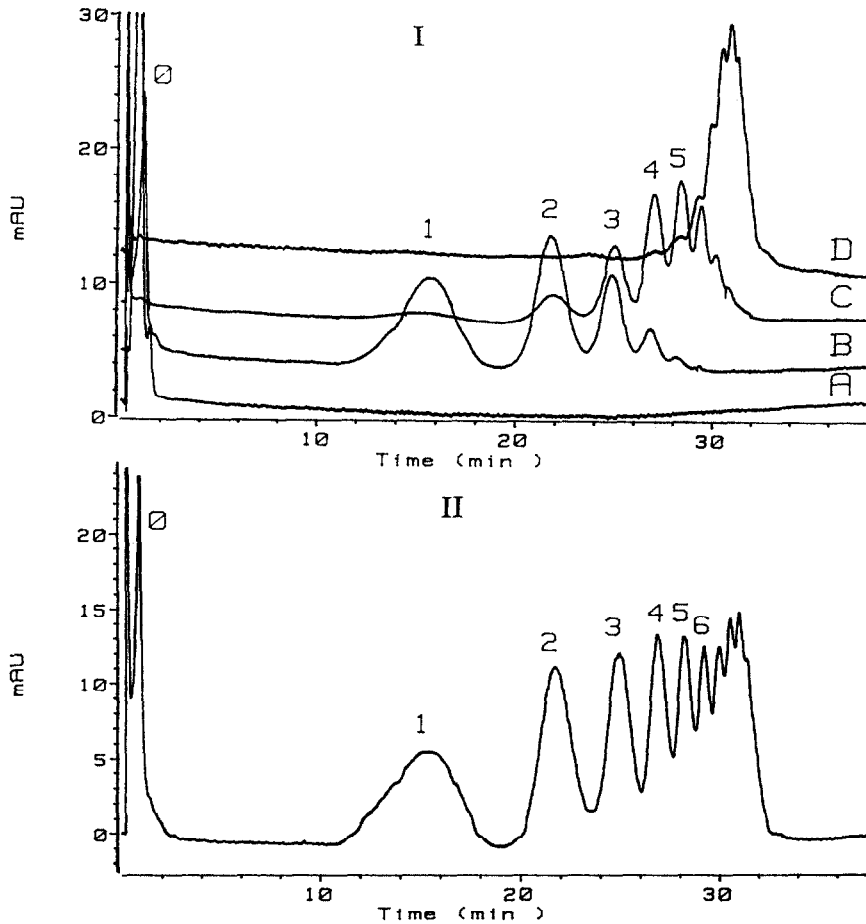

Fig. 9. HPHIC chromatograms of different samples of PEG–SOD. (I): Chromatograms of (A) unmodified SOD, (B) 1-min PEG–SOD sample, (C) 4-min PEG–SOD sample and (D) 50-min PEG–SOD sample. (II): Chromatogram of a mixture of the samples in (I). Numbers over the peaks refer to the predicted number of PEG chains per SOD dimer.
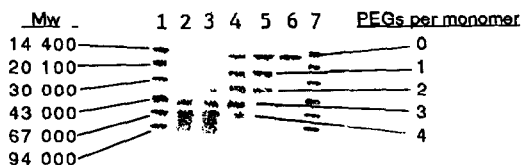
Fig. 10. SDS-PAGE analysis of different samples of PEG–SOD. Lanes: 1 and 7 = molecular weight (Mw) markers; 2 = production scale sample of PEG–SOD; 3 = 50-min PEG–SOD; 4 = 4-min PEG–SOD; 5 = 1-min PEG–SOD; 6 = unmodified SOD.

fore, the quantitative aspects of this method are in some doubt.

IEF

The results of IEF analysis of the PEG-SOD samples are shown in Fig. 11. SOD and two other bands, believed to be the mono- and diPEG–SOD species, are separated from the rest of the reaction mixture. However, the resolution of the remaining PEG–SOD species drops off dramatically as the degree of PEG modification of SOD increases. The pore size of the gel is not suspected of causing this loss of resolution since agarose gels, which typically contain much larger pore sizes than can be obtained using polyacrylamide gels, provided similar results. More likely, the addition of more than 3–4 PEG units shield the charge on the protein thereby masking charge differences observed for the highly PEG modified compounds.

Two bands were observed in the laboratory-scale preparation samples that were not observed in the larger, production-scale preparations of PEG–SOD. The p*I* values of these extra bands are 4.4 and 4.6. Preliminary evidence suggests these extra bands are due to the residual PEG reagents in the PEG–SOD samples.
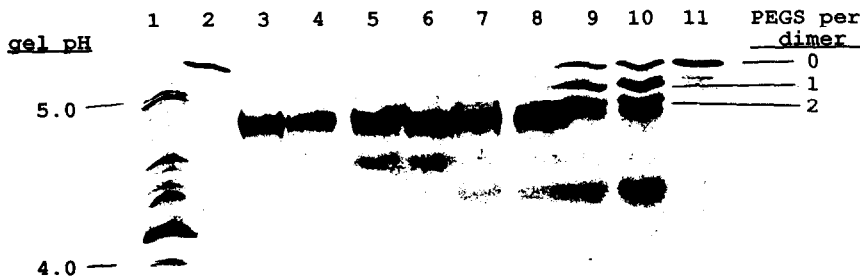
Other investigators have reported that native SOD is not homogeneous, citing the appearance of several bands using disc gel electrophoresis and/or IEF [21,22]. The SOD sample used for the present study was determined to contain a few minor components as evidenced by SDS-PAGE, IEF and CZE; however, the levels of these components were quite low in comparison with the major SOD component. Therefore, to demonstrate the homogeneity of the native SOD used in the PEG–SOD reaction and to confirm that the appearance of additional bands/peaks in the PEG–SOD samples were due to the formation of different PEG–SOD species as opposed to other variants of native SOD, SOD controls were run with each analytical technique. In each case, only minor secondary components, if any, were observed for the SOD controls.

CZE

Given the high resolving power that has been demonstrated in several instances for the CZE analysis of proteins, it was expected that this technique would be ideally suited to the analysis of a complex material such as PEG–SOD. In fact, the characterization of PEG–SOD is somewhat analogous to the characterization of the heterogeneity of glycoproteins which have been successfully analyzed by CZE [24,25]. However, the initial results did not meet expectations. Fig. 12 shows a series of free solution CZE electropherograms obtained on the different samples of PEG–SOD used in this work. The use of a high pH buffer (10.1) along with an electroosmotic flow rate modifier (1,3-diaminopropane) was required to achieve separation of individual species. Again, the breadth of the PEG–SOD peaks, which is in stark contrast to the sharp peak for unmodified SOD, is believed to be due to the wide distribution

Fig. 11. IEF analysis of different samples of PEG–SOD. Lanes: 1 = p*I* markers; 2 and 11 = unmodified SOD; 3 and 4 = production-scale samples of PEG–SOD; 5 and 6 = 50-min PEG–SOD; 7 and 8 = 4-min PEG–SOD; 9 and 10 = 1-min PEG–SOD.
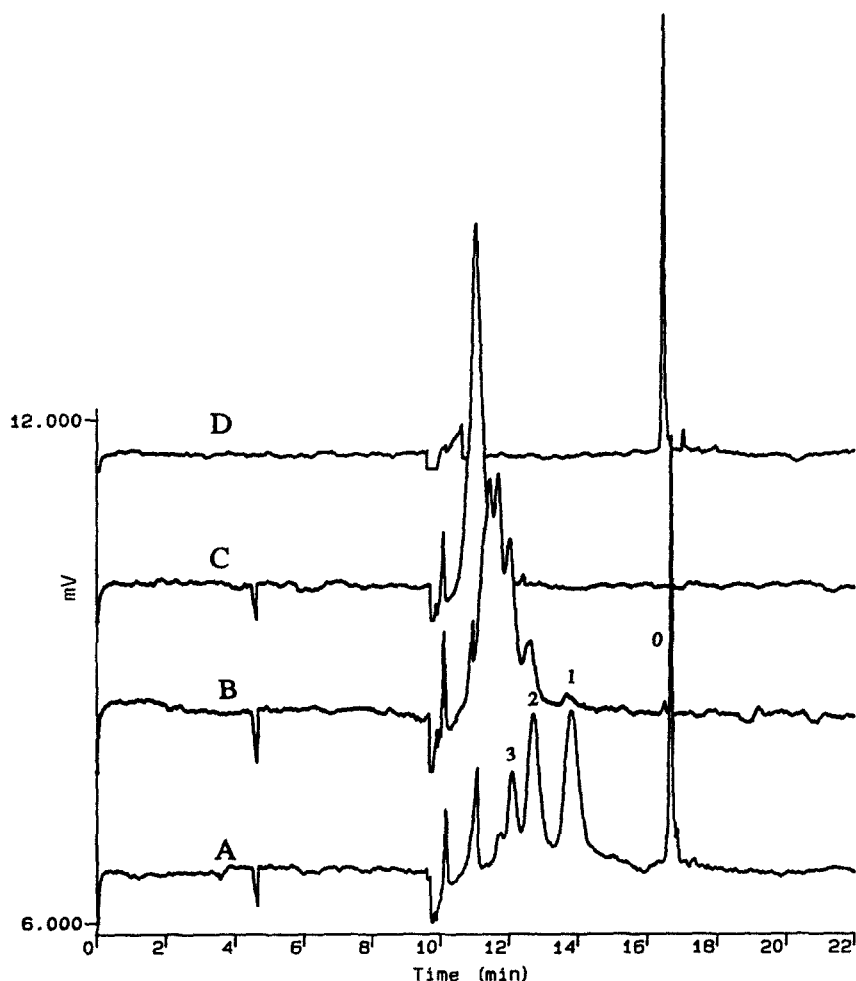
Fig. 12. Free solution CZE analyses of different PEG–SOD samples. (A) 1-min sample, (B) 4-min sample, (C) 50-min sample and (D) unmodified SOD. Data collected using CZE system B. Capillary: 100 cm × 50 μm I.D. (Separation distance 70 cm). Buffer: 15 mM boric acid, 15 mM 1,3-diaminopropane, 10 mM NaCl, pH 10.1. 30 kV, 8-s hydrodynamic injection at a height of 7 cm (2 mg/ml), detection at 200 nm. Numbers over the peaks refer to the predicted number of PEG chains per SOD dimer.

of species possible for a given stoichiometric combination of PEG with SOD. However, the resolution achieved here could not match that achieved by the chromatographic methods.

Fig. 13 contains a series of electropherograms obtained on these same samples using a buffer containing SDS. The use of SDS results in a significant increase in resolution for the lower percent modified SOD samples. This is not believed to be a micellar separation mechanism since these modified proteins are believed to be too large to partition into an SDS micelle. Instead, the enhancement in resolution is

thought to be due to a difference in the binding of SDS among the various PEG modified SOD species. That is, the PEG binds to a greater extent than does the polypeptide chain of PEG–SOD. This would explain the reversal in migration order witnessed here compared to results obtained in the absence of SDS. A strong association of SDS with PEG type polymers (*ca.* 1.6 g SDS/g PEG) has been documented elsewhere [23].

The generally lower-than-expected degree of resolution of PEG–SOD by free solution CZE prompted investigation of alternative ways to ana-
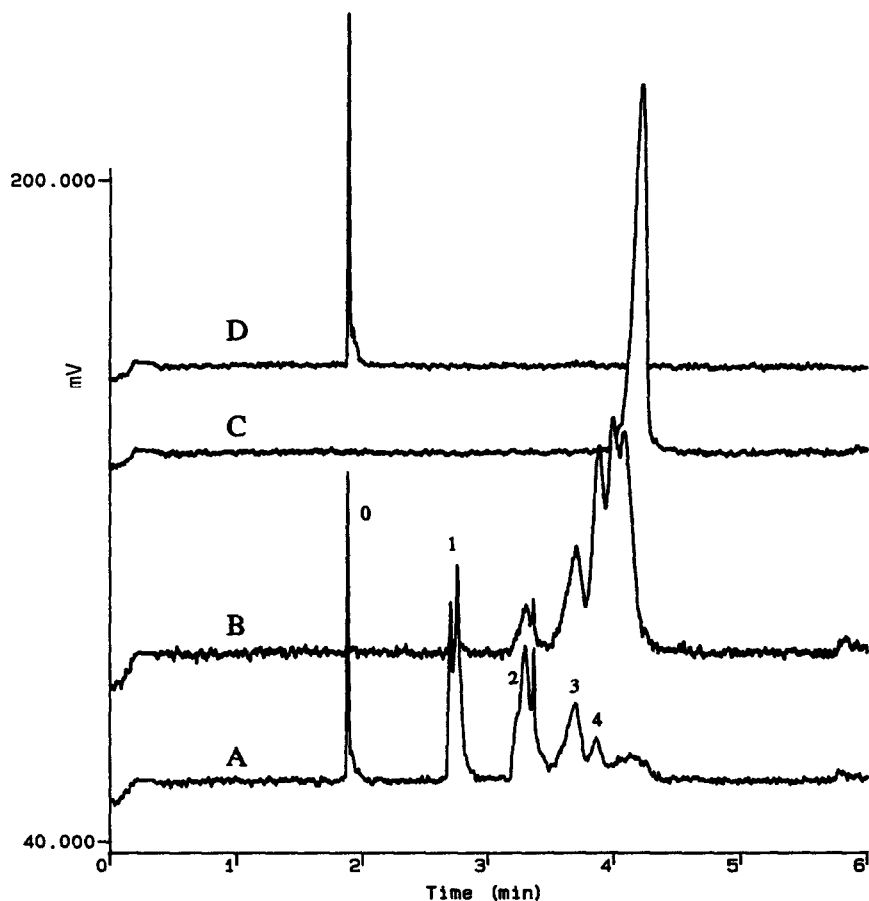
Fig. 13. CZE analyses of different PEG–SOD samples in the presence of SDS. (A) 1-min sample, (B) 4-min sample, (C) 50-min sample and (D) unmodified SOD. Numbers over peaks represent the predicted number of PEG chains per SOD dimer. Data collected on CZE system A. Capillary: 47 cm × 50 μm (separation distance 40 cm). Buffer: 20 mM boric acid, 50 mM SDS pH 9.3. 30 kV, 1-s pressure injections (1 mg/ml) with detection at 200 nm.

lyze the samples. It is possible that the PEG groups are masking charge differences in the samples or it could be that the high degree of heterogeneity in both the mass and charge of the product results in a broad distribution of closely eluting incompletely resolved peaks. In order to simplify this problem, an attempt was made to remove the PEG from the PEG–SOD by hydrolysis prior to analysis by CZE. In this way the material would retain its charge differences (i.e. PEG modified lysines remain amidated while the mass differences in the species would be nearly eliminated. Fig. 14 shows electropherograms of the samples of PEG–SOD after treatment with a borate buffer at pH 10.3. A SOD control was treat-

ed in the same way to demonstrate that there would be no significant change to the polypeptide chain. Significantly better resolution of individual species is achieved upon removal of the PEG groups. The peak at 8.4 min in the 1- and 4-min PEG–SOD samples is due to glycine succinate formed by hydrolysis of the quenched PEG reagent.

A close examination of these electropherograms shows that the later eluting peaks (higher degree of modification) seem to display anomalously high peak broadening (assuming diffusion limited peak broadening). Apparently, this broadening reflects a degree of heterogeneity that still exists for a given stoichiometric combination of PEG with SOD.
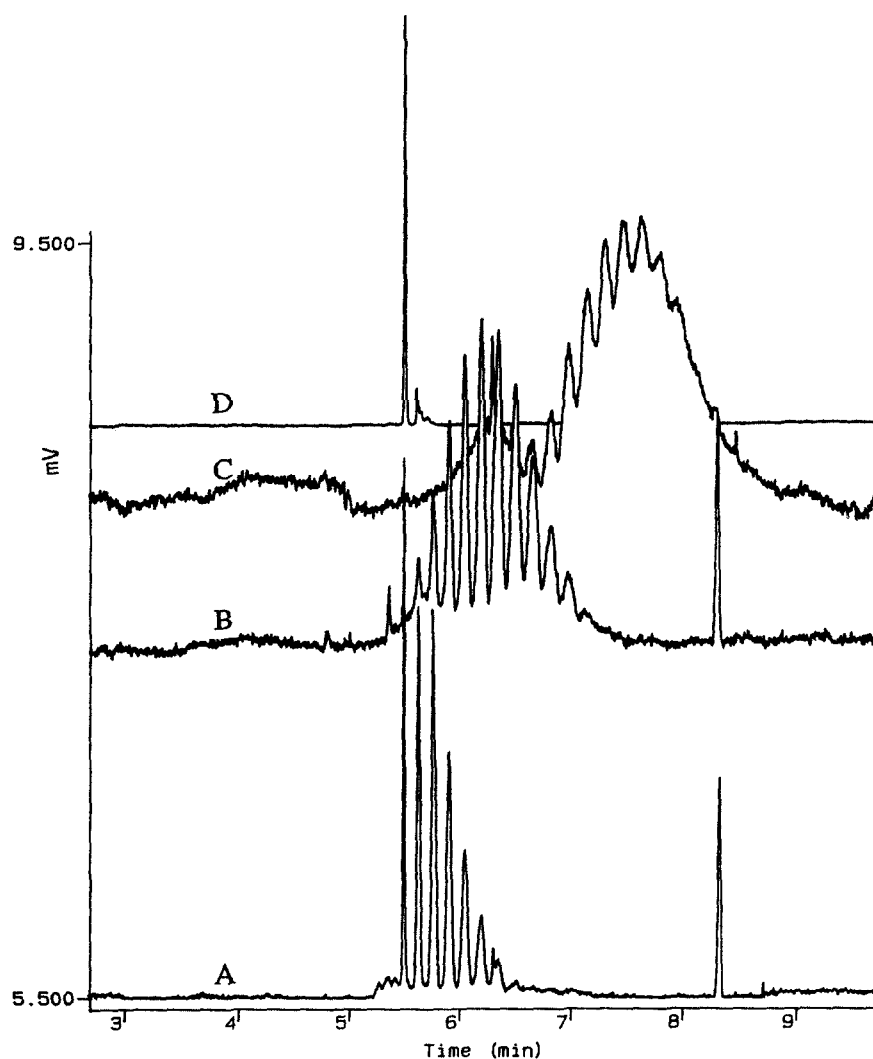
Fig. 14. Free solution CZE analyses of different PEG–SOD samples after removal of the PEG chains. (A) 1-min PEG–SOD sample, (B) 4-min PEG–SOD sample, (C) 50-min PEG–SOD sample and (D) SOD control. Data collected using CZE system B. Capillary: 85 cm long × 50 μm I.D. (separation distance 70 cm). Buffer: 50 mM boric acid, pH 9.0 with NaOH. 30 kV, 40–10-s hydrodynamic injections at a height of 7 cm (1 mg/ml), detection at 200 nm.

This is not unexpected since the lysines on SOD will have slightly different pK_a values depending on their position along the polypeptide backbone as well as their local environment and so are not all equivalent. The higher the nominal degree of modification the larger is the number of possible combinations of modified lysines which correlates into broader peaks.

CONCLUSIONS

Modification of proteins by covalent attachment of PEG chains produces a complex product which displays heterogeneity in both the size and charge of the resulting species. A series of chromatographic and electrophoretic techniques have been developed which have shown the ability to separate and quan-

titate individual species. These methods should have utility in characterizing the reaction of PEG with SOD under different conditions as well as serving as a qualitative tool for verifying the consistency of the product from lot-to-lot.

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