ELSEVIER

Biochimica et Biophysica Acta 1570 (2002) 165–173

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.bba-direct.com

The role of poly(ethyleneimine) in stabilization against metal-catalyzed oxidation of proteins: a case study with lactate dehydrogenase

Javier D. Breccia[1], Maria M. Andersson[2], Rajni Hatti-Kaul*

Department of Biotechnology, Center for Chemistry and Chemical Engineering, Lund University, Getingevagen 60, P.O. Box 124, S-221 00 Lund, Sweden

Received 19 September 2001; received in revised form 24 January 2002; accepted 4 February 2002

Abstract

The protection provided by poly(ethyleneimine) (PEI) to muscle lactate dehydrogenase (LDH) in metal-catalyzed oxidation (MCO) systems ($CuSO_4$ or $FeCl_2$ combined with H_2O_2) was studied, and comparisons were made with the chelators EDTA and desferal, respectively. The analytical chelating capacity of PEI was estimated to be around 1 mol Cu^{2+}/10 mol ethyleneimine for all molecular weights of the polymer. The effect of [PEI monomer]/[metal ion] molar ratio on the oxidatively induced aggregation of LDH exhibited a similar trend as that of the other chelators; aggregation was enhanced at lower ratios and subsequently decreased until it was undetectable with increasing ratio. In contrast, the LDH activity showed a monotonic increase with increasing concentrations of the chelator. Total protection to the enzyme by PEI was provided at concentrations lower than that needed for full chelation of the copper ions, i.e. at [PEI monomer]/[Cu^{2+}] ratio above 9 in case of PEI 2000, and above 7 for PEI 25 000 and 2.6×10^6, respectively. The polymer also provided protection against oxidation in an iron-based MCO system. Hydroxyl radical formation during the MCO reaction was inhibited in the presence of PEI. The polymer of higher molecular weights also exhibited a stronger free radical scavenging effect.

Keywords: Protein stabilization; Metal-catalyzed oxidation; Aggregation; Poly(ethyleneimine); Lactate dehydrogenase; Free radical

1. Introduction

Metal-catalyzed oxidation (MCO) has been recognized to be an important cause of inactivation of therapeutic proteins and peptides during bioprocessing and storage, and is also associated with tissue damage during aging and various pathological states including neurodegenerative disorders [1–5]. MCO-induced damage to proteins is primarily irreversible, and has been the subject of several studies in order to understand the underlying reaction mechanism [3,6–12].

MCO reactions are generally regarded as site-specific processes wherein the metal ions bind to metal-binding sites on the protein, and then react with hydrogen peroxide to yield an active oxygen species (hydroxyl radical (HO•), the superoxide anion (O_2^{-}•), peroxyl radical, etc.), which preferentially attack the amino acid residues located in close vicinity [6]. The reactive intermediates vary with the metal to peroxide concentration ratio as well as with temperature, pH and the presence of chelating compounds, thus yielding a very complex picture of the oxidation process [7,13,14]. The hydroxyl radical (HO•), generated by reaction of the metal with peroxide (Fenton reaction), is believed to be the most reactive and damaging among the oxygen radicals [7,11,13–15]. The overall process is visualized as a ''caged'' reaction and is insensitive to inhibition by radical scavengers [6]. Nonspecific oxidation can also occur by radicals generated by the MCO reaction wherein the radicals formed in solution first have to travel the distance to the protein surface before they randomly cause oxidation [11]. These radicals can be removed by a scavenging compound before they reach the site of oxidative attack.

Chelating agents are commonly used as additives to suppress the inactivation of proteins by MCO reactions [14,15]. However, the effectiveness of a chelator in preventing protein damage is seen to depend on the identity of the metal ion and the chelator to metal ratio [14]. The choice of

* Corresponding author. Tel.: +46-46-222-4840; fax: +46-46-222-4713.
E-mail address: Rajni.Hatti-Kaul@biotek.lu.se (R. Hatti-Kaul).
[1] Present address: PROIMI, Av. Belgrano y Pasaje, Caseros, 4000 Tucuman, Argentina.
[2] Present address: Department of Chemical Engineering, University of California, Berkeley, CA 94720, USA.

0304-4165/02/$ - see front matter
PII: S0304-4165(02)00193-9

a suitable chelator thus becomes important since the amount required for protein protection can be unexpectedly high while significant damage can occur at lower chelator/metal ratios. Some metal–chelator complexes, e.g. ethylenediaminetetraacetate (EDTA)–Fe^{2+} produce greater amounts of radicals and at faster rates than the unchelated metals [7,11]. In such cases, the protein may be lacking a high-affinity site for metal ions but can bind the chelator–metal ion complex, which will react with hydrogen peroxide in a site-specific manner and generate radical species in the microenvironment of the protein [6].

Lactate dehydrogenase (LDH) is an enzyme known to be sensitive to MCO, and has thus been used as a model protein in several studies dealing with the subject [12–14,16,17]. In our earlier study, we observed drastic reduction in the content of cysteine thiols and tryptophan residues, total loss of secondary structure, and also aggregation of the enzyme in the presence of 100 μM $CuSO_4$ only [17]. Addition of only H_2O_2 (10 mM), on the other hand, did not lead to any significant structural changes even though enzyme activity was lost. We also reported stabilization of LDH against oxidation by a cationic polymer, poly(ethyleneimine) (PEI) [17]. The polymer was superior to a range of other chemical additives including EDTA used in the study, in preventing loss of enzyme activity and secondary structure, and aggregation. Its effect was proposed to largely originate from its metal-chelating property [18].

The present study reports the effect of PEI against oxidation of LDH in MCO systems containing Cu^{2+}/Fe^{2+} ions and hydrogen peroxide. The protein oxidation was primarily monitored by its influence on protein aggregation.

2. Materials and methods

2.1. Chemicals

All the chemicals used were of analytical grade and were obtained from standard sources. PEI (50% aqueous solution) of average molecular weights ca. 2000 and 25 000, and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS) were purchased from Aldrich Chemical (Milwaukee, USA). PEI of high molecular weight (50% aqueous solution, $M_w \sim 2.6 \times 10^6$ according to our measurements using static light scattering [19]), desferrioxamine mesylate (desferal), L-LDH (porcine muscle; solution in 50% glycerol (7.3 mg/ml) EC 1.1.1.27), horseradish peroxidase (Type II, 240 units/mg), pyruvic acid (sodium salt), and β-nicotinamide adenine dinucleotide, reduced form, disodium salt (NADH) were procured from Sigma Chemical (St. Louis, MO).

2.2. Determination of Cu^{2+} ion chelating capacity of PEI

A 30 μM solution of $CuSO_4$ was titrated with increasing concentrations of PEI 2000, 25 000 and 2.6×10^6, respectively, in a total volume of 1 ml. The absorbance of the solutions was read at 273 nm in a spectrophotometer (Ultrospec 1000, Pharmacia Biotech).

2.3. Oxidation of LDH

A stock solution of LDH was prepared by dialysing overnight the commercial enzyme solution against deionised water and then diluting to a concentration of 1.5 mg/ml in 50 mM Tris–HCl buffer pH 7.2. For oxidation of the enzyme, 200 μl of LDH solution (50 or 100 μg/ml in 25 mM Tris–HCl buffer pH 7.2) was incubated at 25 °C in presence of the respective MCO reactants, and a chelator (PEI/EDTA/desferal). The copper-based MCO solution consisted of 20 μM $CuSO_4$ and 20 μM H_2O_2, and the iron-based system contained 200 μM $FeCl_2$ and 20 mM H_2O_2. All buffers used were first treated with Chelex 100 (placed in a dialysis bag) for 24 h to remove free metal ions.

2.4. Determination of protein aggregation

Two hundred microliters of LDH samples (50 or 100 μg/ml) containing the various MCO reactants and chelators were distributed in the wells of a microtiterplate. Aggregation of the protein during incubation of the samples at 25 °C was followed every 30 min as an increase in optical density at 620 nm on a microplate reader (DigiScan reader, ASYS Hitech GmbH, Austria).

2.5. Hydrogen peroxide determination

The levels of H_2O_2 in the copper-based MCO system containing 0.1 mM $CuSO_4$ and 20 mM H_2O_2, and varying concentrations of PEI in 1 ml of 50 mM Tris–HCl buffer, pH 7.2, were measured according to an established procedure [11]. To 50 μl of sample in a microtiter plate was added 200 μl of reagent consisting of 10 mM phenol, 0.74 mM 4-aminoantipyrine, 1.5 μg/ml of horseradish peroxidase in 100 mM potassium phosphate buffer, pH 7.0. After incubation for 15 min at 37 °C, the absorbance of the colored product formed was measured at 550 nm in the microplate reader.

2.6. Determination of hydroxyl radical formation

The effect of PEI on HO• radical formation during MCO was monitored by measuring the benzoate hydroxylation as reported earlier [11,20]. A 5 ml reaction mixture containing 0.1 mM $CuSO_4$ (or 0.2 mM $FeCl_2$), 20 mM H_2O_2, 1 mM benzoate and different concentrations of PEI 2000 and 25 000, respectively, in 50 mM Tris–HCl buffer, pH 7.2 was incubated for 24 h at 25 °C. The increase in fluorescence using excitation wavelength of 308 nm and emission wavelength of 410 nm was measured at different time intervals. To express fluorescence in terms of salicylic acid equivalents produced on hydroxylation of benzoic acid

during the reaction, a standard curve of salicylic acid (0–6 μM) was constructed.

2.7. Determination of $ABTS^{+}$ radical scavenging effect of PEI

The ability of PEI to scavenge free radicals was also determined by using an $ABTS^{\bullet+}$ radical cation as a model [21]. Comparison was made with the effect of EDTA. $ABTS^{\bullet+}$ radical cation was prepared by passing a 5 mM stock solution of ABTS through manganese dioxide powder on a Whatman No. 3 filter paper. The filtrate was passed through a 0.2 μm syringe filter to remove excess MnO_2. This solution was then diluted in 5 mM phosphate-buffered saline (PBS) pH 7.4 to give an absorbance of around 0.7 at 734 nm. Aliquots (0–20 μl) of PEI (0.1% w/v) or EDTA (10 mM) were added to 1.0 ml of $ABTS^{\bullet+}$ solution and the mixture was vortexed for 30 s. The reduction in absorbance at 734 nm with time was followed in a spectrophotometer.

2.8. Measurement of LDH activity

The activity of LDH samples was measured according to the established procedure [22] modified for performing the assay in a microtiterplate reader. To the reaction mixture composed of 100 μl of 0.1 M Tris–HCl buffer (pH 7.5), 7 μl of 30 mM pyruvate and 7 μl of 6.6 mM NADH in each well, was added 10 μl of suitably diluted enzyme solution. The initial rate of consumption of NADH at room temperature was followed spectrophotometrically at 340 nm. One unit of LDH activity was defined as the amount of enzyme causing the oxidation of 1 μmol of NADH per minute under the specified conditions.

2.9. Measurement of protein concentration

Total protein concentration of LDH used for the experiments was determined by the bicinchoninic acid method [23]. Bovine serum albumin was used as the standard protein.

3. Results

3.1. MCO and aggregation of LDH

In our earlier study, aggregation of LDH was observed during oxidation in the presence of Cu^{2+} ions and was monitored as optical density at 620 nm [17]. Fig. 1 shows that incubation of the enzyme with 20 μM $CuSO_4$ led to an increase in OD after an initial lag period of about 1 h. However, no change in optical density was observed in the presence of Fe^{2+} or H_2O_2. Aggregation was further used as the main parameter for following oxidation in MCO systems comprising Cu^{2+}/Fe^{2+} and H_2O_2. The peroxide was added to the solution after the metal salt since it has been observed

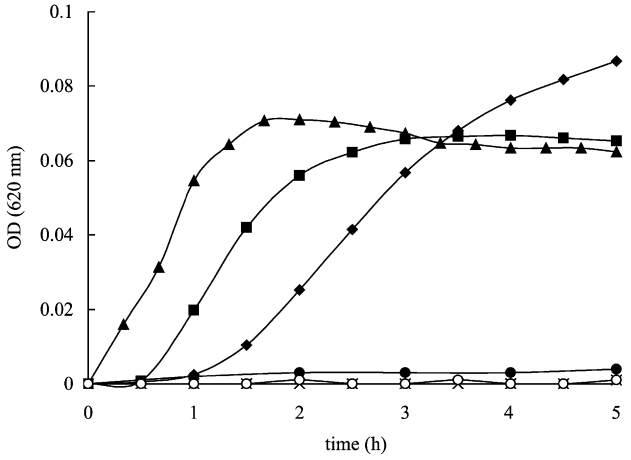

Fig. 1. Time course of aggregation of LDH at 25 °C in samples containing enzyme only (○), and enzyme with 20 μM $CuSO_4$ (◆), 20 μM $CuSO_4$ and 20 μM H_2O_2 (■), 200 μM $FeCl_2$ and 200 mM H_2O_2 (▲), 200 μM $FeCl_2$ (●) and 20 μM H_2O_2 (×). The concentration of the enzyme was 50 and 100 μg/ml in the copper- and iron-containing systems, respectively.

that the amino acid oxidation is much slower and in some cases significantly delayed if the order of addition is reversed [8,24]. Supplementation of the LDH sample containing 20 μM $CuSO_4$ with 20 μM H_2O_2 accelerated the initial rate of aggregation and reached a constant level within a few hours in contrast to the sample with Cu^{2+} alone, which showed a gradual increase in OD for a longer time period. In case of the iron-based system, concentrations of 200 μM $FeCl_2$ and 20 mM H_2O_2 were required to obtain almost similar degree of aggregation (Fig. 1).

3.2. Effect of PEI on LDH oxidation in the copper-based MCO system

3.2.1. Cu^{2+}-chelating capacity of PEI

Fig. 2 shows the copper-chelating capacity of different molecular weights of PEI, measured by determining the absorbance of the complex at 273 nm. Although the complex also has an absorbance peak in the visible region at 600 nm, higher concentrations of the components are needed to detect it [25]. Wavelength scans of a solution containing 30 μM $CuSO_4$ and increasing concentrations of PEI (M_w 2000, 25 000 and 2.6×10^6) revealed an increase in absorbance of the PEI–Cu^{2+} complex until the [PEI monomer]/[Cu^{2+}] ratio was around 10 (Fig. 2). Furthermore, no displacement in the peak at 273 nm was detected indicating the formation of similar complex with the PEI of different molecular weights. At higher PEI concentrations, no further increase in absorbance was noted due to the depletion of free copper ions in solution.

3.2.2. Aggregation and activity of LDH vs. PEI concentration

The influence of PEI on the extent of aggregation and activity of LDH in a Cu^{2+}–H_2O_2 system was compared with

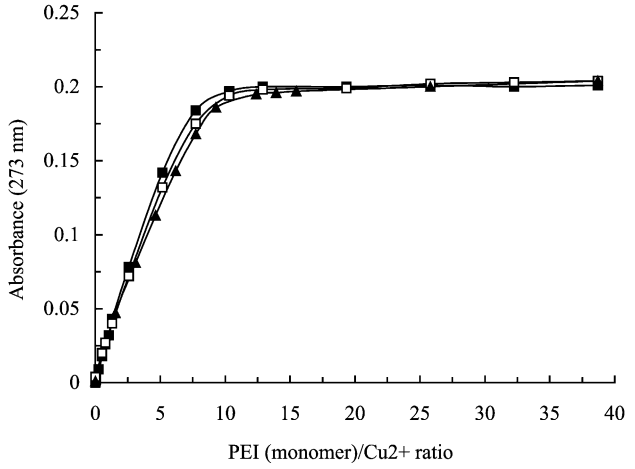

Fig. 2. Titration of 30 μM $CuSO_4$ solution with PEI of different molecular weights at 25 °C. Symbols: ▲ PEI 2000; □ PEI 25 000; ■ PEI 2.6×10^6. See details in Materials and methods.

that of EDTA. In both cases, aggregation of LDH was enhanced at low [chelator]/[Cu^{2+}] ratios, and subsequently decreased with increasing ratio (Fig. 3). PEI of molecular weights 25 000 and 2.6×10^6 acted very similar in providing enhanced aggregation at [PEI monomer]/[Cu^{2+}] ratios below 0.5, and no aggregation at ratios above 7 (Fig. 3a). PEI 2000 showed a somewhat different behavior in that it did not have any effect on the aggregation at [PEI monomer]/[Cu^{2+}] ratios below 1.5, enhanced aggregation was observed at ratios between 1.5 and 5, and no aggregation at ratios above 9 (Fig. 3a). In contrast, in the EDTA containing sample, the aggregation of LDH was reduced at [EDTA]/[Cu^{2+}] ratios above 1 and was undetectable at ratios above 2.7 (Fig. 3b).

The residual activity of the enzyme was measured after 30 min of incubation at which time LDH had lost substantial part of its catalytic activity in the absence of the chelators but was not aggregated. Fig. 4 shows the residual activity of LDH in the presence of different concentrations of PEI 2000 and EDTA, respectively. The activity profile resembled a rectangular hyperbola without revealing any region of maximal inactivation. Full protection of LDH activity was reached at [PEI monomer]/[Cu^{2+}] and [EDTA]/[Cu^{2+}] ratios of around 7–10 and 1–2, respectively, showing good agreement with the aggregation behavior. PEI 25 000 and 2.6×10^6 showed a similar effect on the enzyme activity as PEI 2000 (not shown).

3.2.3. Aggregation vs. varying H_2O_2 to metal ratio

The aggregation of LDH was followed with time at fixed copper and PEI concentrations but with varying peroxide concentration. The [PEI monomer]/[Cu^{2+}] ratio chosen for PEI 2000 was 7.0, and for PEI 25 000 and PEI 2.6×10^6 was 1.2, i.e. a range where the polymers provided only a partial but similar degree of protection against aggregation in a solution containing 20 μM H_2O_2 (Fig. 3a). Fig. 5 shows surface plots of protein aggregation followed with time with respect to the [H_2O_2]/[Cu^{2+}] ratio. At low concentration of H_2O_2, PEI 2000 showed a better protection during a longer time due to the higher [PEI monomer]/[Cu^{2+}] ratio as compared to that of the higher molecular weight polyethyleneimines. However, PEI 2000 was not so effective as the other two when the H_2O_2 concentration was increased to [H_2O_2]/[Cu^{2+}] ratio of 5 and above. The LDH aggregation was initiated earlier with increase in H_2O_2 concentrations, and a defined zone of maximum aggregation was obtained in the

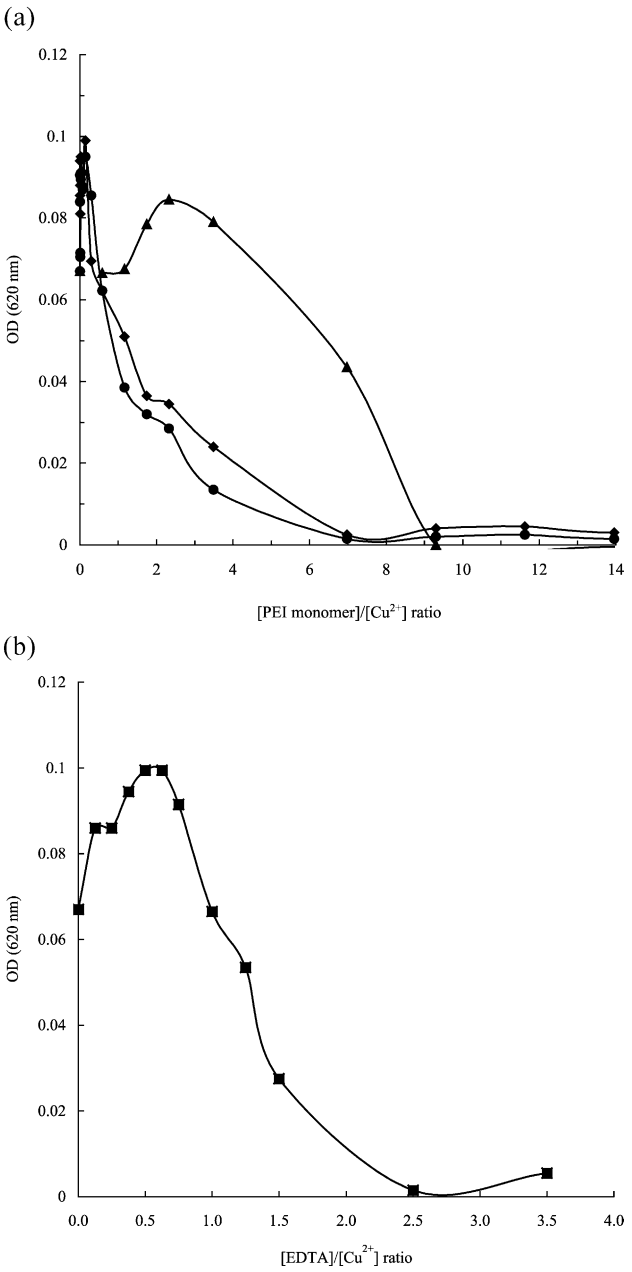

Fig. 3. Effect of the concentration of (a) PEI of different molecular weights, PEI 2000 (▲), PEI 25 000 (♦), and PEI 2.6×10^6 (●), and (b) EDTA on the aggregation of lactate dehydrogenase after 4-h incubation in the MCO system, 20 μM $CuSO_4$ and 20 μM H_2O_2 at 25 °C. Experimental details are described in the text.

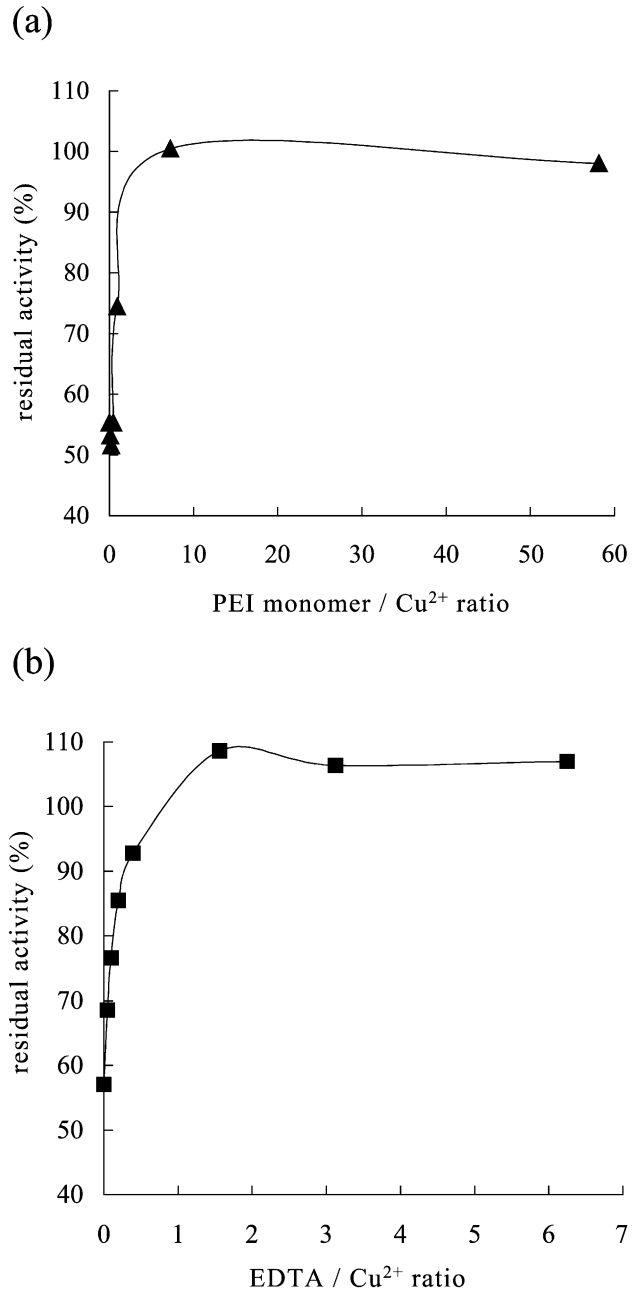

Fig. 4. Effect of the concentration of (a) PEI 2000 and (b) EDTA on LDH activity after 30-min incubation in presence of 20 μM $CuSO_4$ and 20 μM H_2O_2 at 25 °C.

$[H_2O_2]/[Cu^{2+}]$ range of 25–400 for PEI 2000 and 20–200 for PEI of higher molecular weights (Fig. 5). Further increase in concentrations of the peroxide resulted in lower aggregation levels.

3.3. Effect of PEI on oxidation of LDH in the iron-based MCO system

The effect of PEI on enzyme aggregation in an iron-based MCO system was also studied and compared to that of the iron chelator, desferrioxamine mesylate (desferal). As can be seen in Fig. 6, the aggregation followed a similar trend as with the chelators in the copper-based MCO system (Fig. 3), enhanced aggregation at lower concentrations of both PEI and desferal, and lowered aggregation at higher chelator concentrations. In contrast to the copper-based system, however, the influence of PEI of all three molecular weights was rather similar, all providing enhancement in aggregation at PEI monomer to iron ratios below 0.1 and full protection at

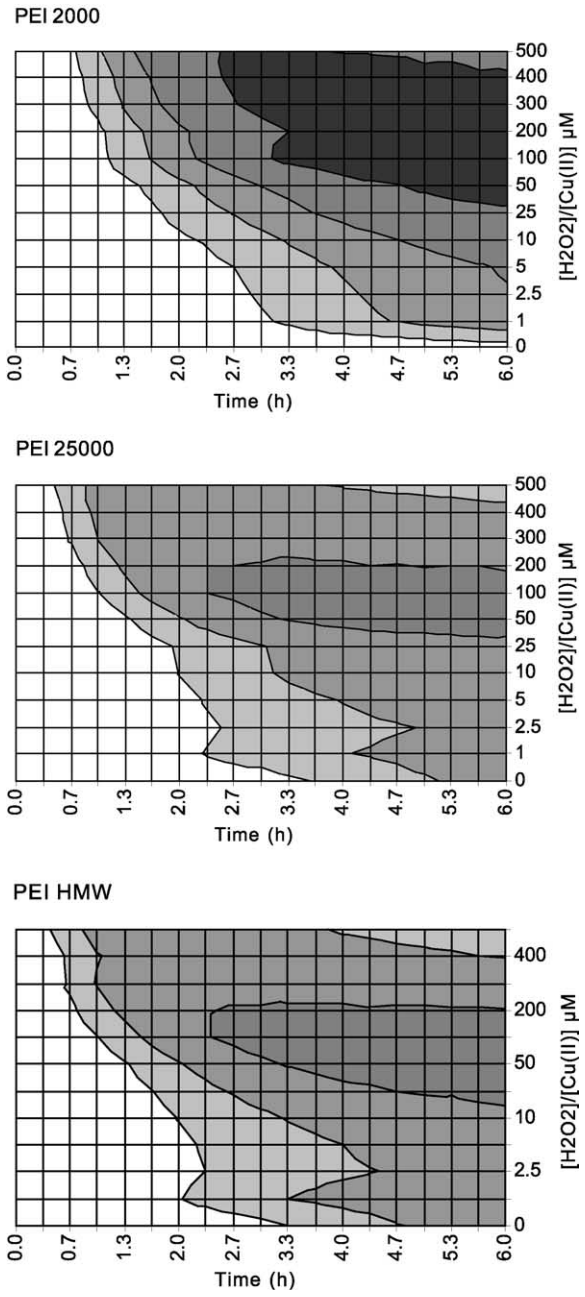

Fig. 5. Surface plot of aggregation of LDH detected by $OD_{620\ nm}$ at different H_2O_2 concentrations at 25 °C. Optical density range: (▭) 0.040–0.060,(▭) 0.060–0.080, (▭) 0.080–0.100, (▭) 0.100–0.120, (▭) 0.120–0.140.

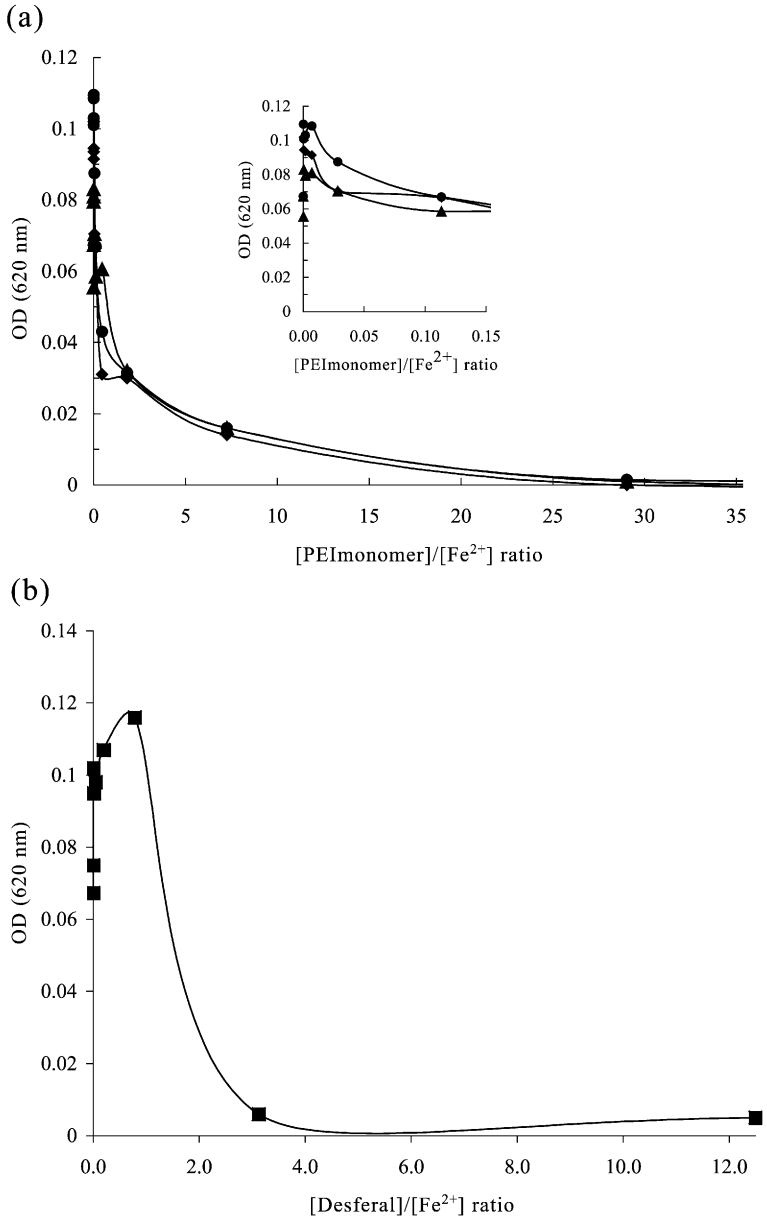

Fig. 6. Effect of the concentration of (a) PEI of different molecular weights, PEI 2000 (▲), PEI 25000 (♦), and PEI 2.6×10^6 (●), and (b) desferal on the aggregation of lactate dehydrogenase after 4-h incubation in the MCO system, 200 μM $FeCl_2$ and 200 mM H_2O_2 at 25 °C. See text for experimental details.

ratios above 29. In the case of desferal, an enhancement in aggregation was observed below a desferal to iron ratio of 2, and near full protection was reached around a ratio of 5.

3.4. Effect of PEI on H_2O_2 consumption and hydroxyl radical formation during MCO reaction

The effect of PEI on H_2O_2 levels and generation of HO• radicals during an MCO reaction was monitored in the absence of the protein. As shown in Fig. 7, the peroxide concentration in a reaction mixture containing 0.1 mM $CuSO_4$ and 20 mM H_2O_2 was reduced with time to about 50% of the original concentration at 24 h. The extent of reduction in H_2O_2 levels was significantly lowered in the presence of increasing PEI concentrations. The change in the peroxide concentration was marginal in the presence of PEI 25 000 even at [PEI monomer]/[Cu^{2+}] ratio of 5.

The formation of hydroxyl radicals under similar conditions was followed by measuring the fluorescent products of benzoate. While benzoate hydroxylation increased significantly with time, it was suppressed by increasing concentrations of the polymer (Fig. 8). PEI 25 000 was again shown to be more effective than PEI 2000 when used at [PEI monomer]/[Cu^{2+}] ratio of 5, while at the ratio of 10 and above, both PEI 2000 and 25 000 strongly inhibited benzoate hydroxylation. Preliminary studies with Fe^{2+}-cata-

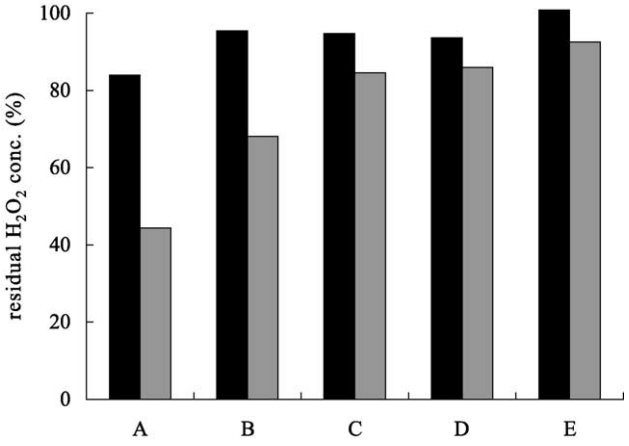

Fig. 7. Effect of PEI on consumption of H_2O_2 in a metal-catalyzed oxidation reaction after 4 h (black bar) and 24 h (grey bar) of incubation at 25 °C. Different samples were (A) 0.1 mM $CuSO_4$ and 20 mM H_2O_2 only; (B,C) metal salt and peroxide with PEI 2000 at [PEI monomer]/[Cu^{2+}] ratio of 5 and 10, respectively; and (C,D) with PEI 25 000 at [PEI monomer]/[Cu^{2+}] ratio of 5 and 10, respectively.

lyzed oxidation showed that the increase in benzoate hydroxylation was much lower and was suppressed in the presence of PEI (not shown).

3.5. Free radical scavenging effect of PEI

The free radical scavenging effect of PEI was further examined with ABTS•$^+$ radical as model. Fig. 9 shows the decrease in absorbance of the ABTS•$^+$ radicals with time after addition of 0.1% PEI to the solution. It can be seen that PEI 2000 has a slower scavenging effect of the free radicals than the polymer having higher molecular weights. If one compares the scavenging effect of PEI and EDTA 3 min after mixing the solution, it turns out that, based on monomer concentration, PEI 2000 was 1.5 times, and PEI 25 000 and 2.6×10^6 were 3 times more efficient as scavengers than EDTA.

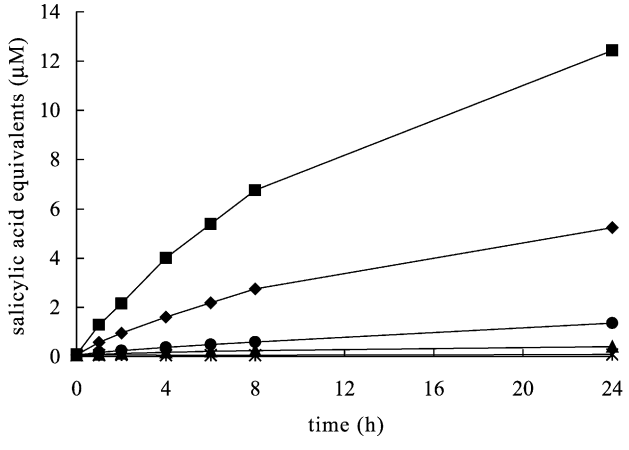

Fig. 8. Effect of PEI on hydroxyl radical formation detected as benzoate hydroxylation. Different samples contained 0.1 mM $CuSO_4$ and 20 mM H_2O_2 only (■), with PEI 2000 at [PEI monomer]/[Cu^{2+}] ratio of 5 (♦) and 10 (▲), and with PEI 25 000 at [PEI monomer]/[Cu^{2+}] ratio of 5 (●) and 10 (×), respectively. The fluorescence intensity is expressed in terms of equivalent salicylic acid produced on hydroxylation of benzoate.

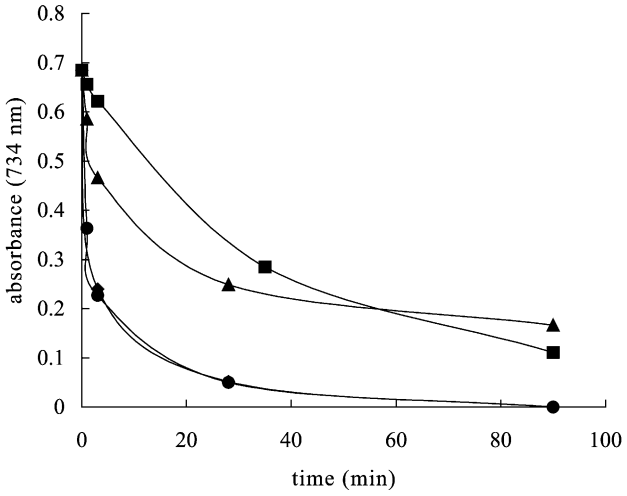

Fig. 9. ABTS radical ion scavenging effect of (▲) PEI 2000, (♦) PEI 25 000, (●) PEI 2.6×10^6 and (■) EDTA.

4. Discussion

Chelating agents exert their protective effect against MCO by removing the metal ions into "bulk" solution, i.e. away from the microenvironment of the protein. The HO• radicals formed in bulk may be more prone to react with nearby media components than with the protein [14]. However, the chelating agents vary in their ability to provide stabilization to the protein. For example, while preventing the formation of HO• radicals and subsequent oxidation via the Cu^{2+}-catalyzed Fenton reaction, EDTA enhances the effect in an Fe^{2+}-catalyzed reaction, resulting in a faster degradation of the protein [11]. On the other hand, desferal was found to be effective in preventing the build up of HO• radicals and stabilizing LDH in an iron-based and not the copper-based MCO system [14].

In this study, we evaluated the effect of PEI during MCO of LDH, which was compared with that of EDTA and desferal, respectively. The latter two compounds form 1:1 complexes with metal ions [8]. The repetitive ethylamine units of PEI render to the polymer the ability to act as a multidentate ligand for metal ions [18]. This property was considered the main basis for stabilization provided by the polymer against protein oxidation during storage [17,26]. The chelating capacity of the polymer was determined to be 1 mol copper ion/10 mol ethyleneimine and was independent of the PEI molecular weight (Fig. 2).

Although HO• radicals are the damaging components during oxidation, measuring the amount of radicals is not considered a reliable indicator of protein damage; only their absence proves the lack of damage [14]. However, protein degradation or aggregation that is often the ultimate result of

the attack of the HO• radicals on amino acids is monitored using electrophoretic and chromatography techniques to evaluate protein damage [10–12,16,20,27–29]. We have used earlier dynamic light scattering and optical density at 620 nm, respectively, to demonstrate aggregation of LDH and protective effect of PEI on the enzyme [17,19]. OD_{620} turned out to be a simple, reproducible and sensitive method for use in the present work.

Aggregation of the enzyme was observed simply on oxidation by air when catalyzed by $CuSO_4$ (Fig. 1). While combination of the metal salt with peroxide led to an increase in the rate of oxidation and aggregation, the extent of aggregation seemed to be relatively higher in the former case. This could probably be due to the different pathways of oxidation in the two cases. In contrast to Cu^{2+}, oxidation with only Fe^{2+} revealed little or no aggregation of LDH. A significantly higher concentration of $FeCl_2$ and peroxide, and also a two times higher LDH concentration, was required in the case of iron-catalyzed oxidation system to give aggregation comparable to that in the copper-based system (Fig. 1). These observations were consistent with earlier studies showing that generation of hydroxyl radicals and site-specific damage of proteins is significant when Cu^{2+} ions are added to hydrogen peroxide but is only marginal in case of Fe^{2+} (or Fe^{3+}) oxidation systems [11].

The influence of the increasing PEI concentration on aggregation and activity of LDH in MCO system containing 20 μM Cu^{2+} and 20 μM peroxide followed a similar trend as that of EDTA. While aggregation was enhanced at low chelator/metal ratio and subsequently decreased to undetectable level with increasing ratio, the residual activity profile showed a monotonic increase with increasing chelator–metal ratio until a maximum was reached corresponding to full protection of the enzyme (Figs. 3 and 4). Despite the different activity and aggregation profiles, the chelator concentration showing total protection of the enzyme was the same. It may be noted that LDH activity was measured only 30 min after initiation of the MCO reaction as it is affected relatively quickly in time scale. The loss of enzyme activity has already been initiated by binding of metal ions at or near the active site (e.g. histidine residue) and immediate modification of critical amino acid(s) by the free radicals released [30]. Aggregation, on the other hand, is a relatively slow process involving the modification of the protein at several sites and eventual cross-linking [6,9]. However, it seemed to reflect the profile of free radicals more closely than the activity loss. An initial increase followed by reduction in aggregation with increasing chelator concentration could be attributed to a similar trend in the amount of HO• radicals produced as reported earlier [14]. Although this trend was not followed in this study, it was observed that at a [PEI monomer]/[Cu^{2+}] ratio of 5, free radicals were indeed accumulated with time (Fig. 8) and continued to damage the protein as seen in Fig. 3. The lower level of HO• formation in presence of PEI 25 000 is consistent with its higher protective effect than that of PEI 2000 (Fig. 3). At a ratio of 10, both 2000 and 25 000 prevented HO• accumulation with the result of no aggregation and retention of activity (Figs. 3 and 4).

Aggregation was further seen to first increase and then decrease with increasing peroxide concentration at fixed metal and PEI concentration (Fig. 5). A similar trend in aggregation or activity with change in peroxide concentration has been reported earlier for other proteins [3,13,24]. The change in aggregation could again be related to the amount of HO• radicals produced, which has been shown to reach a maximum with increasing peroxide concentration and subsequently decrease due to the scavenging action of the peroxide itself at higher concentration [13]. The region of maximum aggregation was reached at a higher peroxide concentration range for PEI 25 000 and 2.6×10^6 than for PEI 2000 (Fig. 5).

Protein aggregation in the EDTA-containing MCO system was eliminated only at [EDTA]/[Cu^{2+}] ratios of 2.7 (Fig. 3b), in accordance with the earlier reports showing that complete inhibition of oxidation occurs at chelator concentration higher than that required to chelate the metal ions totally [8,14]. Desferal exhibited a similar effect in the Fe^{2+}–H_2O_2 system, providing total protection at chelator/metal ratio of 5 (Fig. 6). On the contrary, PEI was effective at concentrations lower than that required for total chelation of copper ions. No aggregation was observed at [PEI monomer]/[Cu^{2+}] ratio above 9 in case of PEI 2000, and above 7 for PEI 25 000 and 2.6×10^6, respectively. Inhibition of HO• radicals formation by PEI further supported these results (Fig. 8). It is thus evident that additional feature(s) not related to the metal-chelating property of the polymers have to be involved in PEI-induced stabilization. Better radical scavenging capacity of the polymer, particularly that of PEI 25 000 and 2.6×10^6 (Fig. 9), could be a very likely explanation. Moreover, dynamic light scattering studies had earlier led us to suggest that a higher molecular weight polyethyleneimine forms a shell around the LDH molecule [19]. This results in reduced protein–protein contacts and hence lower degree of aggregation.

5. Conclusions

Chelating agents have an undisputed application as stabilizers of protein formulations since oxidation constitutes a major cause of inactivation. Such compounds may also have therapeutic values for the treatment, e.g. of neurodegenerative disorders, which are initiated by oxidation-related protein aggregation. This study shows that polyethyleneimine protects protein against both copper- and iron-based oxidations in contrast to the chelating agents conventionally used, which are more selective in their protective effect. Moreover, due to its other interesting features like antimicrobial effect [31] and potential as agent in controlled release [32], PEI can turn out to be a promising alternative to other chelating agents for preventing protein oxidation.

Acknowledgements

The work was supported by a financial grant from the Swedish International Development Cooperation Agency (Sida).

References

[1] E.R. Stadtman, Science 257 (1992) 1220–1224.
[2] B.S. Berlett, E.R. Stadtman, J. Biol. Chem. 272 (1997) 20313–20316.
[3] S.R. Paik, H.-J. Shin, J.-H. Lee, Arch. Biochem. Biophys. 378 (2000) 269–277.
[4] D.A. Butterfield, J. Kanski, Mech. Ageing Dev. 122 (2001) 945–962.
[5] M.Y. Aksenov, M.V. Aksenova, D.A. Butterfield, J.W. Geddes, W.R. Markesbery, Neuroscience 103 (2001) 373–383.
[6] E.R. Stadtman, Free Radic. Biol. Med. 9 (1990) 315–325.
[7] I. Yamazaki, L.H. Piette, J. Biol. Chem. 265 (1990) 13589–13594.
[8] E.R. Stadtman, B.S. Berlett, J. Biol. Chem. 266 (1991) 17201–17211.
[9] E.R. Stadtman, Annu. Rev. Biochem. 62 (1993) 797–821.
[10] T. Ogino, S. Okada, Biochim. Biophys. Acta 1245 (1995) 359–365.
[11] T. Kocha, M. Yamaguchi, H. Ohtaki, T. Fukuda, T. Aoyagi, Biochim. Biophys. Acta 1337 (1997) 319–326.
[12] K.D. Bush, J.A. Lumpkin, Biotechnol. Prog. 14 (1998) 943–950.
[13] R.D. Madurawe, J.A. Lumpkin, Chem. Eng. Commun. 162 (1997) 23–44.
[14] R.D. Madurawe, Z. Lin, P.K. Dryden, J.A. Lumpkin, Biotechnol. Prog. 13 (1997) 179–184.
[15] S. Li, C. Schöneich, R.T. Borchardt, Biotechnol. Bioeng. 48 (1995) 490–500.
[16] Alonso-Llamares, de Arriaga D., J. Soler, Biochem. Int. 27 (1992) 879–889.
[17] M.M. Andersson, J.D. Breccia, R. Hatti-Kaul, Biotechnol. Appl. Biochem. 32 (2000) 145–153.
[18] J. Suh, Y. Cho, K.J. Lee, J. Am. Chem. Soc. 113 (1991) 4198–4202.
[19] M.M. Andersson, R. Hatti-Kaul, W. Brown, J. Phys. Chem., B 104 (2000) 3660–3667.
[20] J.V. Hunt, J.A. Simpson, R.T. Dean, Biochem. J. 250 (1988) 87–93.
[21] N.J. Miller, J. Sampson, L.P. Candeias, P.M. Bramley, C.A. Rice-Evans, FEBS Lett. 384 (1996) 240–242.
[22] V. Worthington, Worthington Enzyme Manual; Enzyme and Related Biochemicals, Worthington Biochemical Corporation, Freehold, NJ, USA, 1993, pp. 230–235.
[23] P.K. Smith, R.I. Krohn, G.T. Hermansson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Anal. Biochem. 150 (1985) 76–85.
[24] K. Sigler, G. Gille, V. Vacata, N. Stadler, M. Höfer, Folia Microbiol. 43 (1998) 361–367.
[25] R. Tauler, E. Casassas, Chemom. Intell. Lab. Syst. 14 (1992) 305–317.
[26] M.M. Andersson, R. Hatti-Kaul, J. Biotechnol. 72 (1999) 21–31.
[27] A. Salvi, P.-A. Carrupt, B. Testa, Helv. Chim. Acta 82 (1999) 870–878.
[28] E. Shacter, Drug Metab. Rev. 32 (2000) 307–326.
[29] J.R. Requena, D. Groth, G. Legname, E.R. Stadtman, S.B. Prusiner, R.L. Levine, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 7170–7175.
[30] K. Kimura, S. Sugano, J. Biochem. 112 (1992) 828–833.
[31] J. Bryjak, A. Noworyta, Enzyme Microb. Technol. 16 (1994) 616–620.
[32] T.G. Park, S. Cohen, R. Langer, Pharmacol. Res. 9 (1992) 37–39.