

Hydroperoxide-mediated fragmentation of proteins

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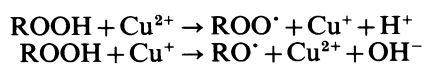
1. Chemiluminescence and benzoic acid hydroxylation were used to detect oxygen-centred free-radical production by 2.5 mM-H₂O₂ and 100 μM-Cu²⁺. Free radicals could not be detected by these methods when H₂O₂ was replaced with 10 mM-t-butyl hydroperoxide (TBH) or 10 mM-cumene hydroperoxide (CH). The inclusion of the thiol compound dithioerythritol (DTET; 100 μM) increased radical production by H₂O₂ and Cu²⁺ as judged by both assays. Mannitol scavenged radicals in the chemiluminescence system in a dose-dependent manner. 2. H₂O₂, TBH and CH, each with Cu²⁺, gave rise to substantial fragmentation of the protein bovine serum albumin (BSA). This fragmentation could be increased by the inclusion of DTET. Omission of Cu²⁺ or the addition of the chelator DETAPAC (diethylenetriaminepenta-acetic acid; 1 mM) lead to virtual abolition of fragmentation. Autoxidized lipid in the presence of Cu²⁺ caused protein fragmentation by reactions of lipid hydroperoxides. 3. Polyacrylamide-gel electrophoresis in the presence of SDS confirmed that production of fragments had occurred. 4. Susceptibility of BSA to enzymic hydrolysis by two different proteinases acting at pH 5 and pH 7.2 was increased after a limited exposure to hydroperoxides in the presence of Cu²⁺. 5. These results may have biological significance, particularly for proteins in lipid environments (e.g. membrane proteins and lipoproteins).

INTRODUCTION

A number of processes known to occur *in vivo* may result, either directly or indirectly, in the formation of damaging free radicals. Examples include the respiratory burst of phagocytes [1], mitochondrial electron transport [2] and metal-catalysed autoxidation of certain substrates such as carbohydrates and unsaturated lipids. One result of such radical formation is the generation of H₂O₂ and various organic hydroperoxides. These in turn, in the presence of transition metals, can produce further radicals. H₂O₂ in the presence of transition metals has been shown to degrade proteins such as those in cartilage [3,4] and BSA [5,6].

The reactions of many hydroperoxides of non-esterified polyunsaturated fatty acids *in vitro*, have been shown to lead to cross-linking of proteins [7]. Peroxidizing phospholipids have been shown to give less cross-linking [8] than peroxidizing non-esterified fatty acids. In contrast, we have reported fragmentation of the mitochondrial membrane-bound enzyme monoamine oxidase in the presence of autoxidizing phospholipid [9]. We present here some studies on the direct action of hydroperoxides in protein fragmentation.

In the presence of transition metals, H₂O₂ is thought to give rise to peroxy radicals and extremely reactive hydroxyl radicals via the Fenton reaction [10]. In the case of organic hydroperoxides (ROOH) an analogous reaction is thought to occur, resulting in the peroxy (ROO[•]) radical and the more reactive alkoxy (RO[•]) radical. With copper as the transition metal [11], the reaction may be expressed as:



The inclusion of thiols, such as DTET, in a reaction mixture consisting of H₂O₂ and Cu²⁺ has been reported to increase free-radical production [12,13]. The effect of some thiol compounds on lipid peroxidation has also been studied, and under certain circumstances they have been reported to increase the rate of peroxidation [14]. Here we demonstrate protein fragmentation by H₂O₂ and certain organic hydroperoxides in the presence of copper. This fragmentation could be increased in the presence of a thiol compound.

MATERIALS AND METHODS

All reagents, unless otherwise stated, were of the highest purity obtainable from Sigma.

Radical detection by Lucigenin chemiluminescence and benzoic acid hydroxylation

Lucigenin-amplified chemiluminescence of a system buffered with 10 mM-potassium phosphate and 4 mM-Hepes, pH 7.2, and containing CuSO₄ (500 μM)/H₂O₂ (5 mM) [15] was used to detect the effect of DTET on the production of radicals for various times up to 3 h. Chemiluminescence was measured at room temperature in a scintillation counter in the out-of-coincidence mode. The effect of various concentrations of the hydroxyl-radical scavenger mannitol were investigated.

Radical detection by benzoic acid hydroxylation was carried out as described in [16]. The increase in fluorescent products with an excitation maximum of 308 nm and emission maximum of 410 nm was measured after 30 min in reaction mixtures kept at 37 °C. Reaction mixtures contained 1 mM-sodium benzoate, 0.1 M-potassium phosphate buffer, pH 7.2, 2.5 mM-H₂O₂ and 100 μM-CuSO₄,

Abbreviations used: BSA, bovine serum albumin; TBH, t-butyl hydroperoxide; CH, cumene hydroperoxide; DTET, dithioerythritol; DETAPAC, diethylenetriaminepenta-acetic acid.

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both with and without 100 μM -DTET and in the presence or absence of BSA (1 mg/ml). The final volume was 3 ml.

To express fluorescence in terms of equivalent salicylic acid produced on hydroxylation of benzoic acid during the reaction, standard curves of a range of concentrations of salicylic acid were constructed. Since some of the components of each reaction mixture investigated affected salicylic acid fluorescence, it was necessary to prepare standard solutions of salicylic acid in the presence of buffer, with and without copper, plus or minus DTET, and in the presence or absence of BSA.

Protein substrate

Freeze-dried Fraction V BSA was obtained from Boehringer Mannheim and was labelled with ^{14}C by reductive methylation with [^{14}C]formaldehyde (Amersham International) as described in [17]. After dialysis for 48 h against distilled water at 0–4 °C, the resulting labelled protein substrate was stored at –20 °C before use.

Fragmentation by hydroperoxides

All reactions were carried out at 37 °C over time courses of 8 h or 2 h, with [^{14}C]BSA (1 mg/ml) at pH 7.2 in 10 mM-phosphate buffer. The final concentration of H_2O_2 used was 2.5 mM, and that of the organic hydroperoxides, TBH and CH, was 10 mM.

The reaction mixtures consisted of an individual hydroperoxide and 100 μM - CuSO_4 , in both the presence and absence of 100 μM -DTET. Controls included BSA in buffer alone or in the presence of hydroperoxide alone. An additional control of the reaction mixture plus 1 mM-DETAPAC was also used.

Enzymic hydrolysis

Substrates for proteolytic studies were prepared by incubating [^{14}C]BSA (10 mg/ml) either alone in phosphate buffer, or with hydroperoxide, with or without Cu^{2+} , in the presence or absence of DTET. The concentrations of reagents other than BSA were identical with those described above for studies on fragmentation.

Incubations were carried out at 37 °C for only 30 min, after which 1 mM-DETAPAC was added to stop the reaction by chelating the metal. In the case of H_2O_2 , catalase was also added; the addition of catalase to end the reaction differed from that in fragmentation studies (see below), in that a lower concentration of catalase [10^{-7} M], with an incubation at room temperature for 60 min was used, to avoid the addition of a significant amount of intact protein. The mixtures were then dialysed as described for reductive methylation. After dialysis, the substrates, all of which had less than 0.8% trichloroacetic acid-soluble ^{14}C -labelled material, were stored at –70 °C before use in enzymic hydrolysis.

Enzymic hydrolysis of the substrate at a concentration of 1 mg of BSA/ml by trypsin (2.5 $\mu\text{g}/\text{ml}$) was allowed to proceed for 1 h at 37 °C in 50 mM-phosphate buffer, pH 7.2. The extent of protein degradation was determined at several time points during the 1 h incubation period. The hydrolysis was repeated, but with pepsin (2.5 $\mu\text{g}/\text{ml}$) buffered with 50 mM-sodium acetate buffer, pH 5.

Measurement of fragmentation by hydroperoxides and hydrolytic enzymes

Fragmentation by hydroperoxides was determined by preparing a 5% (w/v) trichloroacetic acid supernatant

from the treated protein and measuring the ^{14}C content in a liquid-scintillation counter. Before addition of trichloroacetic acid, the reaction in the sample removed from the reaction mixture was stopped by the addition of a final concentration of 1 mM-DETAPAC and, in the case of H_2O_2 , by incubation with 10 μM -catalase for 15 min at room temperature.

In the case of trypsin, a 5%-(w/v)-trichloroacetic acid supernatant was prepared. After peptic digestion, the enzyme was inactivated by preparation of an alkaline supernatant made by the addition of 100 μl of 25% (w/v) ZnSO_4 , followed by 100 μl of saturated $\text{Ba}(\text{OH})_2$ to 1 ml of the peptic digest.

In all cases percentage fragmentation or percentage enzymic hydrolysis to soluble fragments was calculated as a percentage of the insoluble pellet at the beginning of the reaction.

Lipid-peroxidation-mediated protein fragmentation

The liposomes used were prepared with type VE phosphatidylcholine in 20 mM-phosphate buffer as described by Gregoriadis & Ryman [18].

Autoxidizing phosphatidylcholine liposomes were then prepared by incubating liposomes (20 mg/ml) with 20 mM-phosphate buffer, pH 7.2, and 100 μM - Cu^{2+} at 37 °C for 36 h. These were then diluted by a factor of 2 on use. The resulting reaction mixture, containing 10 mM-phosphate buffer, was adjusted to 1 mg of [^{14}C]BSA/ml, 10 mg of liposomes/ml and 100 μM - Cu^{2+} , and then incubated for 48 h at 37 °C. At several time points protein fragmentation was determined as described above.

Controls used were BSA in buffer alone or the complete mixture with 1 mM-DETAPAC.

SDS/polyacrylamide-gel electrophoresis

Portions of reaction mixtures in which fragmentation occurred and from the control (BSA in buffer alone) were used to provide samples for these studies. After removal of a 1 ml sample from such mixtures, 0.1 ml of DETAPAC was added with mixing (the final concentration being 1 mM) and then 1.1 ml of sample buffer, containing 5% (v/v) dithiothreitol and 2% (w/v) SDS, was then added. In the case of reaction mixtures containing H_2O_2 , catalase was also added 15 mins before the sample buffer. After denaturation by heating at 100 °C for 3 min, the samples were separated on a 10% (w/v) polyacrylamide gel [19] containing 0.1% SDS.

To optimize the detection of any fragments formed during exposure of BSA to hydroperoxides, lanes were heavily loaded (10 μg of BSA/lane), and protein was then stained with a sensitive AgNO_3 stain. The loading of the BSA monomer was such that staining was outside the range in which a linear relation exists between protein concentration in the gel band and the staining intensity [20]. Protein standards included catalase (4 $\mu\text{g}/\text{lane}$) and a mixture of markers (2.4 $\mu\text{g}/\text{lane}$) containing proteins in the M_r range 20 100–68 000.

RESULTS

Radical detection by Lucigenin chemiluminescence and benzoic acid hydroxylation

The effect of DTET on Lucigenin-amplified chemiluminescence in an $\text{H}_2\text{O}_2/\text{Cu}^{2+}$ radical-generating system, as shown in Fig. 1, was to increase the initial rate of

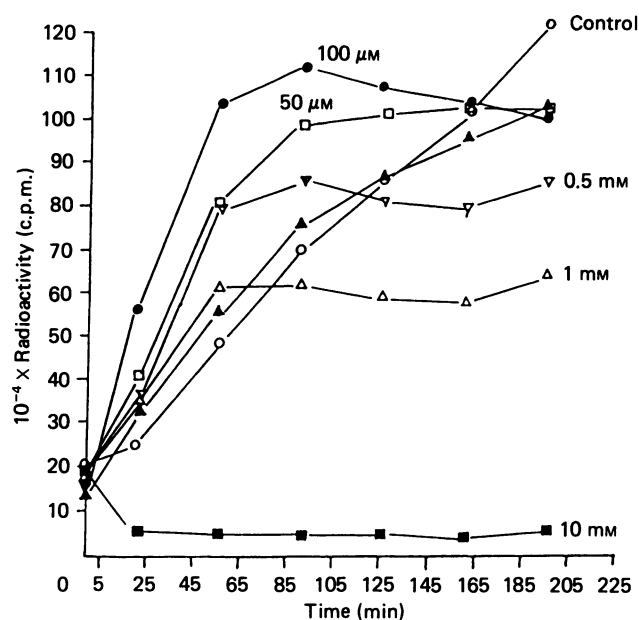


Fig. 1. Dependence on the concentration of dithioerythritol of chemiluminescence generated by $\text{Cu}^{2+}/\text{H}_2\text{O}_2$

Chemiluminescence generated by the incubation of Cu^{2+} ($500 \mu\text{M}$) and H_2O_2 (5 mM) in the presence of various concentrations of DTET: \circ , control (no DTET added); \blacktriangle , $10 \mu\text{M}$; \square , $50 \mu\text{M}$; \bullet , $100 \mu\text{M}$; ∇ , $500 \mu\text{M}$; \triangle , 1 mM ; \blacksquare , 10 mM .

free-radical production at DTET concentrations up to $100 \mu\text{M}$. Higher concentrations quenched the chemiluminescence, presumably by scavenging the radicals. The inclusion of 0.1 mM -, 1 mM -, 10 mM - and 100 mM -mannitol in the same free-radical generating systems gave a dose-dependent inhibition of chemiluminescence, reaching 90% at 100 mM (results not shown).

Table 1. Radical detection by benzoate hydroxylation

Incubation mixtures comprising 1 mM -benzoic acid, 100 mM -potassium phosphate buffer, 2.5 mM - H_2O_2 , $100 \mu\text{M}$ - Cu^{2+} plus or minus $100 \mu\text{M}$ -DTET in the presence and absence of BSA (1 mg/ml) were incubated at 37°C for 30 min, after which time the yield of fluorescent benzoate hydroxylation products was determined in the mixture as a whole. H_2O_2 alone is shown below as an example of several control conditions studied; all the others, lacking H_2O_2 , gave smaller values. All values are means \pm S.D. for three experiments and are significantly different ($P < 0.01$; Student's t test) from each other. The amount of salicylic acid was calculated from relevant standard curves (see the Materials and methods section).

Reaction system	Salicylic acid-equivalents (nmol)
H_2O_2 alone	0.12 ± 0.02
$\text{H}_2\text{O}_2/\text{Cu}^{2+}$	0.38 ± 0.02
$\text{H}_2\text{O}_2/\text{Cu}^{2+}/\text{DTET}$	2.82 ± 0.2
$\text{H}_2\text{O}_2/\text{Cu}^{2+}/\text{BSA}$	0.33 ± 0.02
$\text{H}_2\text{O}_2/\text{Cu}^{2+}/\text{DTET}/\text{BSA}$	0.5 ± 0.03

Studies using benzoic acid hydroxylation confirmed that DTET enhances radical production in both the presence and absence of BSA (1 mg/ml) (see Table 1). The addition of BSA gave a substantial decrease in hydroxylation when compared to an $\text{H}_2\text{O}_2/\text{Cu}^{2+}$ free-radical-generating system without added BSA; this may have been due to radical scavenging.

Neither of these two detection systems were capable of detecting free radicals derived from organic hydroperoxides in incubations up to 30 min (corresponding to the conditions of Table 1).

Fragmentation of [^{14}C]BSA by hydroperoxides

Whether DTET was absent or present, all hydroperoxides studied gave rise to substantial amounts of trichloroacetic acid-soluble ^{14}C -labelled fragments, the formation of which was dependent on the addition of Cu^{2+} (Fig. 2). The addition of the metal chelator DETAPAC totally prevented fragmentation. Very slight fragmentation by hydroperoxides alone, without any added Cu^{2+} , was probably due to contaminating transition metals in the reagents.

Lipid peroxidation-mediated protein fragmentation

Fig. 3 shows that peroxidizing phospholipid can also fragment BSA and that the fragmentation can be prevented, for the greater part, by the addition of DETAPAC. Thus lipid hydroperoxides probably mediate fragmentation in a similar manner to the organic hydroperoxides.

SDS/polyacrylamide-gel electrophoresis of BSA after hydroperoxide attack

Electrophoretic studies, shown in Fig. 4, confirm fragmentation of BSA. In the representative examples shown, it is clear that exposure of BSA to hydroperoxides and Cu^{2+} causes a destruction of the large aggregates present in the commercial sample that migrate near the top of the gels. Thus it seems that none of the hydroperoxides used in the present study gave rise to cross-linking. At the 68 kDa (BSA monomer) position there is the impression of broadening of the stained band after these reactions. This is due to a combination of overloading the staining system and monomer fragmentation to slightly smaller peptides. Fig. 4 also indicates that fragmentation of BSA monomer by the hydroperoxides can lead to the production of a quite heavily staining band of about 48 kDa that migrates slightly more slowly than the 45 kDa standard (ovalbumin). This band is absent (or virtually so) in the control lanes. In the case of H_2O_2 , two other peptides, one slightly bigger and one slightly smaller than the 29 kDa molecular-size marker protein, are also evident. This production of fragments of specific sizes (rather than random sizes) is in agreement with our previous studies on protein fragmentation during defined radical attack [6,9,24] and other data [21,24 (review)]. The difference in the size of fragments observed on exposure of the protein to different hydroperoxides implies a difference in mechanism and/or kinetics of fragmentation. Further studies (results not shown) using a smaller load of protein confirm the implied loss of the 68 kDa monomer and higher-molecular-size polymers as well as the production of a very small amount of the smaller peptides described above.

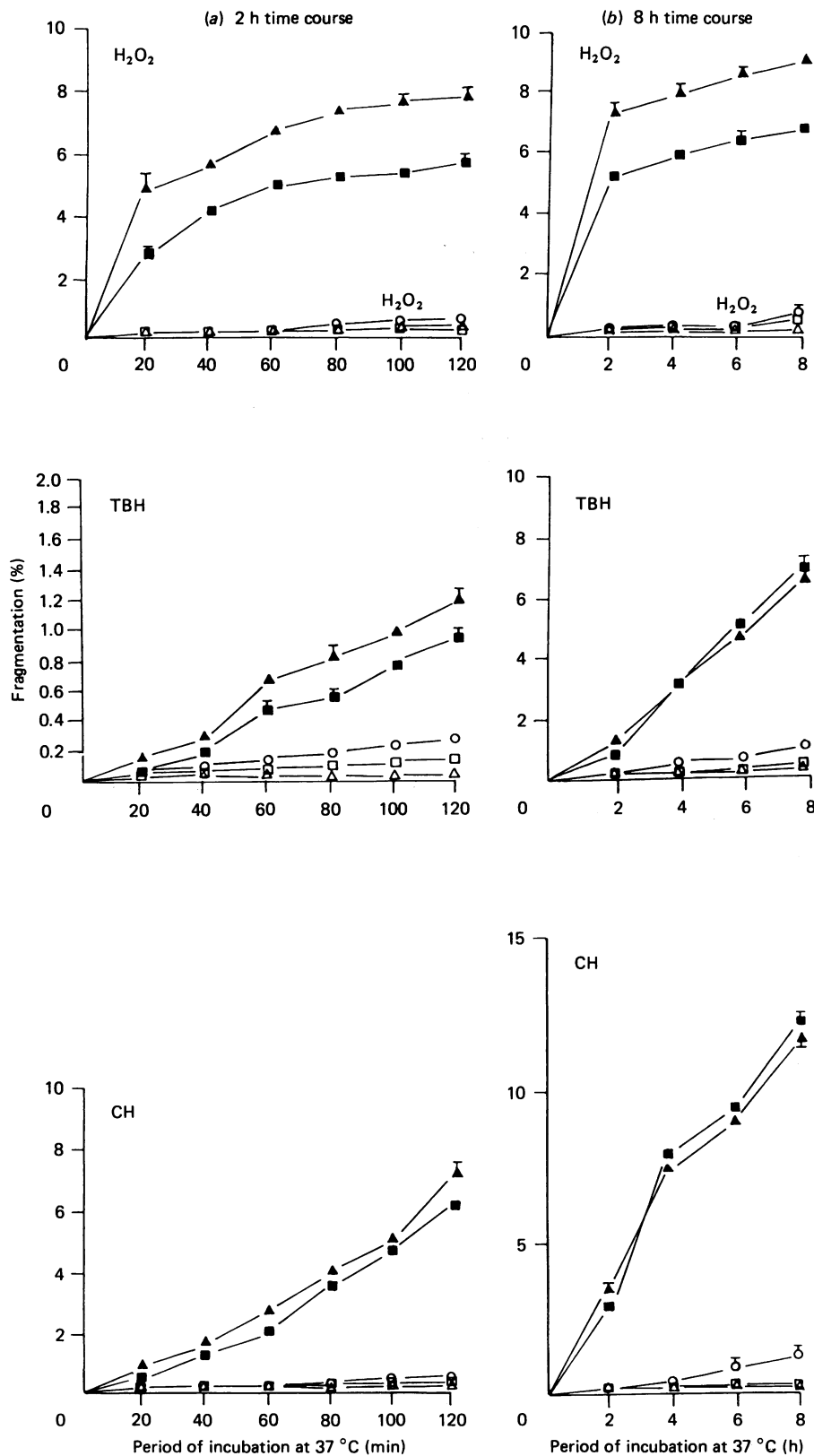


Fig. 2. Hydroperoxide-mediated fragmentation of BSA

Fragmentation, over two time scales, of BSA (1 mg/ml in 10 mM-potassium phosphate buffer, pH 7.2) at 37 °C on exposure to: Δ , buffer alone; \circ , hydroperoxide alone; \blacksquare , hydroperoxide and Cu^{2+} ; \blacktriangle , hydroperoxide, Cu^{2+} (100 μM) with DTET (100 μM); \square , hydroperoxide, Cu^{2+} (100 μM), DTET (100 μM) with DETAPAC (1 mM). Hydroperoxides were used at the following concentrations: H_2O_2 , 2.5 mM; TBH, 10 mM; CH, 10 mM. Each point represents the mean value from two experiments; assays were performed in duplicate in both experiments. Bars indicate the range of values obtained in assays when this exceeds the size of the symbol.

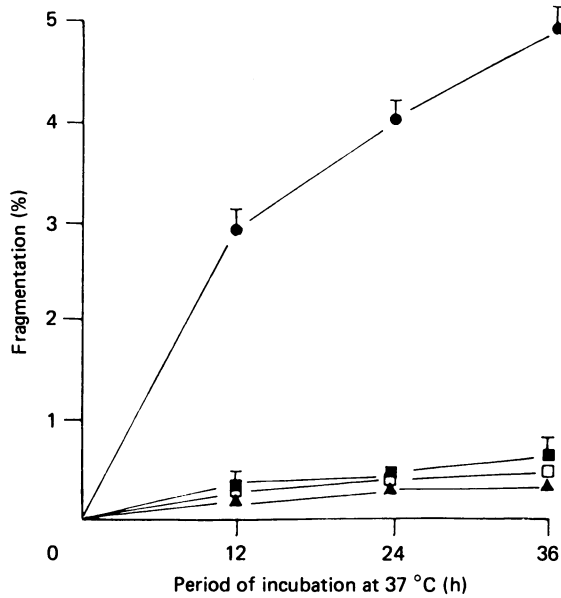


Fig. 3. Fragmentation of BSA by peroxidized lipid

BSA at 1 mg/ml was incubated in 10 mM-phosphate buffer at 37 °C in the presence of: □, buffer alone; ▲, buffer and DETAPAC (1 mM); ●, peroxidized phosphatidylcholine liposomes (10 mg/ml) and Cu²⁺ (100 μM); ■, peroxidized phosphatidylcholine liposomes (10 mg/ml), Cu²⁺ (100 μM) and DETAPAC (1 mM). Each value is the mean for two experiments; assays were performed in duplicate in both experiments. Bars indicate the range of values obtained in assays when this exceeds the size of the symbol.

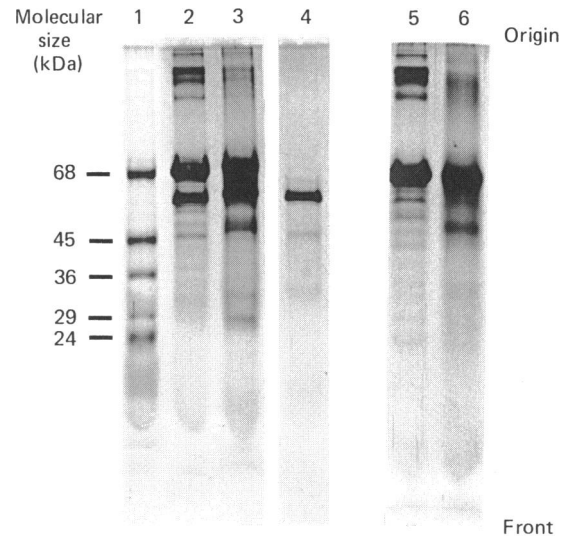


Fig. 4. SDS/polyacrylamide-gel electrophoresis of BSA incubated with hydroperoxides

BSA (1 mg/ml) was exposed to hydroperoxides, at 37 °C and pH 7.2, for a time corresponding to the production of 6%-(w/v)-trichloroacetic acid-soluble radioactive label in the case of H₂O₂, and for 24 h in the case of CH. Lane 1, molecular-size protein markers; lane 4, catalase (which is also present in lanes 2 and 3); lanes 2 and 3 respectively are samples from an experiment with BSA incubated in buffer alone (lane 2), or (lane 3) in the presence of H₂O₂ (2.5 mM) and copper (100 μM). Lanes 5 and 6 are samples from another experiment in which BSA was incubated in buffer alone (lane 5) or (lane 6) in the presence of CH (10 mM) and Cu²⁺ (100 μM).

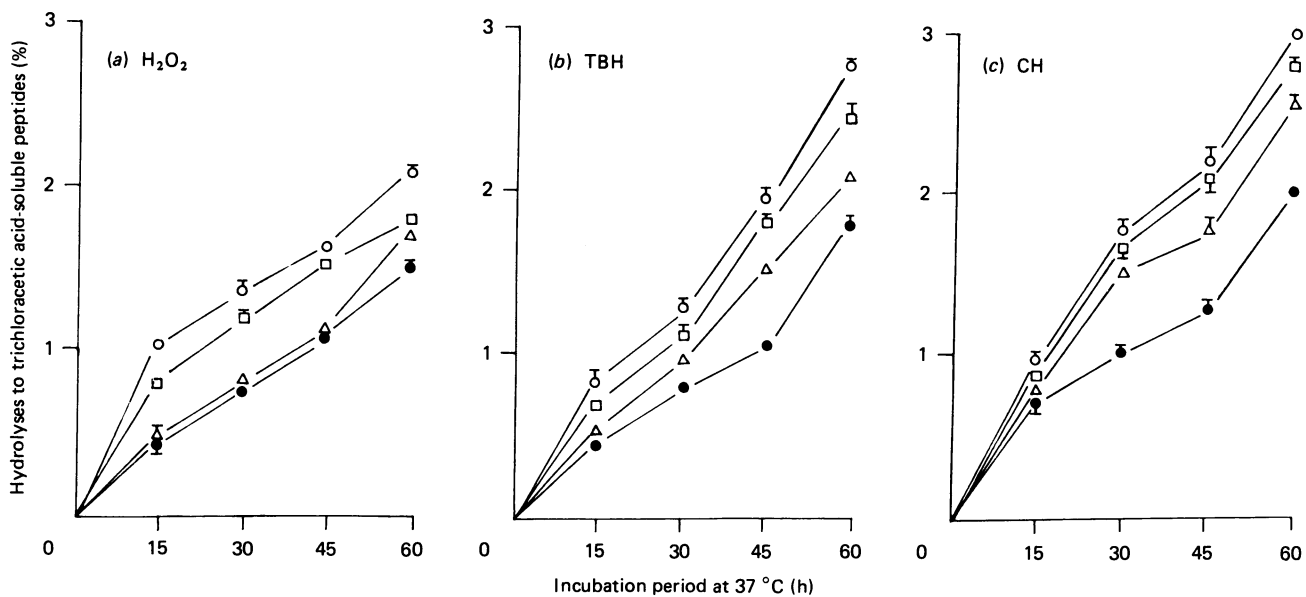


Fig. 5. Increased susceptibility of BSA to tryptic hydrolysis after exposure to hydroperoxides

BSA was preincubated under various conditions then subsequently exposed at a concentration of 1 mg/ml to trypsin (2.5 μg/ml) in phosphate buffer (50 mM, pH 7.2 and 37 °C) for up to 60 min. As detailed in the Materials and methods section, preincubations were at 37 °C for 30 min under the following conditions: ●, BSA (1 mg/ml) in buffer alone; △, with hydroperoxide alone; □, with hydroperoxide and Cu²⁺; ○, with hydroperoxide, Cu²⁺ and DTET. (a) H₂O₂; (b) TBH; (c) CH. Each value is the mean for two experiments; assays were performed in duplicate in both experiments. Bars indicate the range of values obtained in assays when this exceeds the size of the symbol.

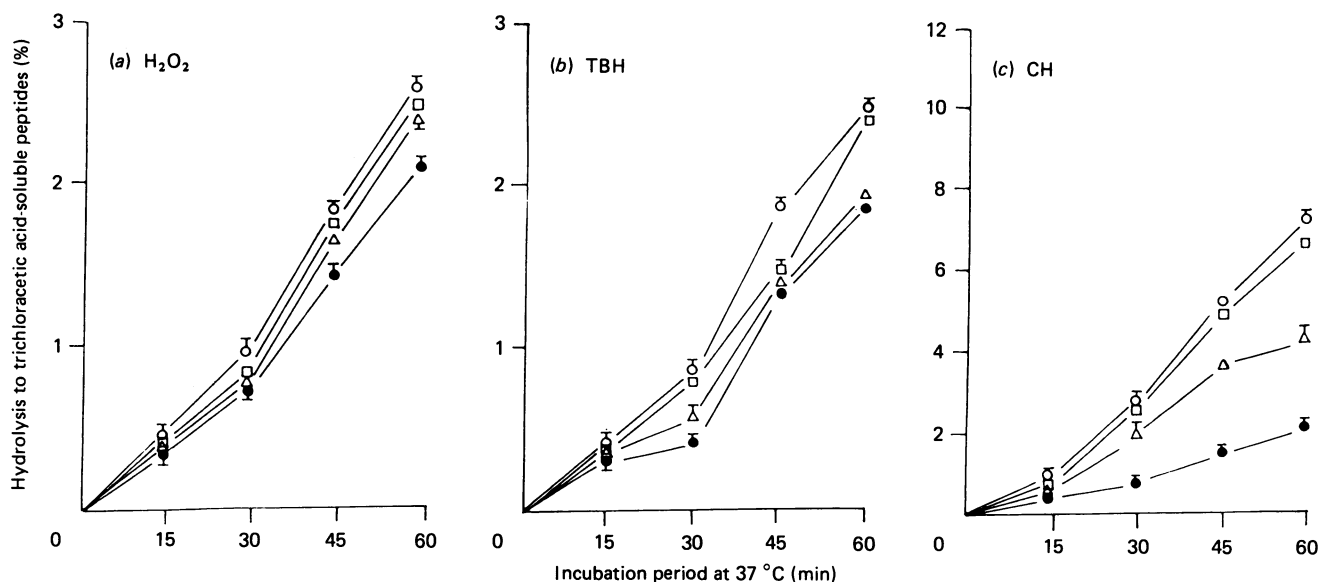


Fig. 6. Increased susceptibility of BSA to peptic hydrolysis after exposure to hydroperoxides

BSA was preincubated under various conditions, then subsequently exposed at a concentration of 1 mg/ml to pepsin (2.5 µg/ml) in acetate buffer (50 mM, pH 5 and 37 °C) for up to 60 min. Preincubations with hydroperoxides were as defined in legend to Fig. 5. (a) H₂O₂; (b) TBH; (c) CH. Each value is the mean for two experiments; assays were performed in duplicate in both experiments. Bars indicate the range of values obtained in assays when this exceeds the size of the symbol.

Enzymic hydrolysis of hydroperoxide-modified BSA

Exposure of BSA to hydroperoxides increased its susceptibility to hydrolysis by trypsin (Fig. 5). The largest increase occurred with exposure to hydroperoxide, Cu²⁺ and DTET, but in each case susceptibility was also increased after exposure to hydroperoxide and metal only. This indicates that increase in susceptibility is dependent, among other factors, on the extent of fragmentation [6]. Similarly, exposure to hydroperoxides and Cu²⁺ with and without DTET slightly increases susceptibility to pepsin (Fig. 6). In this case there is no clear additional effect of DTET beyond that of the hydroperoxide/metal systems. Incubation with the organic hydroperoxides (and particularly CH), in the absence of copper, also slightly facilitates subsequent enzymic hydrolysis. This may be due to the effects of hydrophobic interactions between hydroperoxides and the protein.

DISCUSSION

In the present study systems comprising Cu²⁺ and hydroperoxide have been shown to generate free radicals and to fragment protein. Site-specificity of BSA fragmentation, as suggested in [21], is probably dependent on the antioxidant property of proteins to chelate Cu²⁺ at specific sites [22]. Fragmentation by CH and TBH may also involve site-specific hydrophobic associations between the protein and hydroperoxide concerned. Such interactions may account for differences observed between the various hydroperoxides with respect to changes in enzymic susceptibility of pre-exposed BSA.

In agreement with [12,13], the thiol compound, DTET, gave a greater production of free radicals in the case of H₂O₂ plus DTET and also increased fragmentation by all hydroperoxides at some point in the

course of the reaction. Various thiols (for example glutathione) are present within cells and hence may influence radical reactions involving hydroperoxides in this way. Paradoxically, glutathione also acts as part of a radical-detoxification pathway via glutathione peroxidase. The absence of this enzyme, which may occur under certain pathological situations, may lead to an increase in free-radical production, resulting in further damage.

Many studies concerning autoxidation of non-esterified polyunsaturated fatty acids *in vitro* have implicated protein cross-linking as the main consequence of exposure of protein to peroxidizing lipid [23]. Fragmentation by peroxidized lipid, as observed here by release of ¹⁴C-labelled fragments, may in some circumstances be accompanied by cross-linking. However exposure to the organic hydroperoxides used in the present study did not result in cross-linking.

A limited exposure to radical-generating systems of hydroperoxide and Cu²⁺ increased susceptibility to enzymic hydrolysis by trypsin at pH 7.2 and, to a lesser extent, pepsin at pH 5. Exposure of proteins to hydroperoxides *in vivo*, particularly in the presence of transition metals, may lead to an increase in proteolysis by both extra- and intra-lysosomal proteinases acting at neutral and low pH respectively.

In view of these data, lipid peroxidation may be expected to alter proteins in foodstuffs and also influence protein activity, protein susceptibility to enzymic hydrolysis, and thus protein turnover, in cells and connective tissues [24]. These effects may be of economic importance and also considerably perturb cellular function.

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