LYSOZYME DAMAGE CAUSED BY SECONDARY DEGRADATION PRODUCTS DURING THE AUTOXIDATION PROCESS OF LINOLEIC ACID

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Summary Autoxidized LA is classified into four groups, LA, LAHPO, SP and FP. Lysozyme is inactivated by these products in the increasing order as follows: FP < LA < LAHPO < SP. The effects of these products on the amino acid composition of lysozyme is examined. All kinds of amino acid residues were not damaged until lysozyme was incubated with LA and LAHPO at 45°C for 100 days. The susceptible amino acid residues attacked by the autoxidized products are tryptophan, lysine and histidine. The specific loss of methionine by SP occurs during acid-hydrolysis. The effect of SP was the strongest among the autoxidized products. FP was almost noneffective. The destructive actions of BP, MA and PA were compared with those of autoxidized products. Effects of these compounds did not resemble those of autoxidized products. It was concluded that tryptophan, lysine and histidine residues were specifically attacked by SP.

Considerable studies have been carried out on the effects of autoxidized fatty acids on several enzymes (1), and it has been established that the autoxidized products destroy amino acid residues in protein. Every amino acid of cytochrome c was decomposed by the oxidized linolenic acid at 37°C for a few hours as reported by Desai and TappeL (2). Roubal and TappeL (3) also obtained the same results as described above in the incubation system of autoxidized ethylarachidonate and some kinds of enzymes at 37°C for 5 hr. On the contrary, only three kinds of amino acids of RNase, tyrosine, lysine and histidine, were broken down by the autoxidized ethylarachidonate at 37°C for 120 hr in the report by Chio and Tapel.
Methionine residue in trypsin, pepsin and RNase was specifically affected by LAHPO and SP at 37°C for a short time (40 min) as reported by GAMAGE and MATSUSHITA (5). Recently, BRADDOCK and DUGAN (6) and YANAGITA and SUGANO (7) studied on the effects of autoxidizing linoleate on myosin and albumin, respectively. They reported that only lysine, histidine and methionine residues were destroyed during incubation under a drastic condition of 50°C for 6 days.

These results show the lack of consistent conclusions concerning the damage of amino acid residues in protein by the autoxidized fatty acids. Especially, there was no definite information about the kinds of susceptible amino acid residues to be attacked by the autoxidized products, and also about the species of autoxidized products having destructive effects on amino acid residues. In the present paper, an attempt was made to clarify these problems, and the method of removing autoxidized products from the incubation systems was examined.

It is generally known that many kinds of autoxidized products are produced at the process of autoxidation of LA. These products were classified into four groups (8, 9), LA, LAHPO, SP and FP. BP, MA and PA were also used as the standard reagents for comparisons with the above products. In the incubation systems containing lysozyme and any of the above products or compounds, the susceptible amino acid residues were also investigated. It was concluded that SP was the most destructive to the amino acid residues among the autoxidized products of LA.

**EXPERIMENTAL**

LA was purchased from Nakarai Chem. Co., Ltd. Lysozyme (6× crystallized) and Micrococcus lysodeikticus were obtained from Seikagaku Kogyo Co. LA-U-14C (1.1 mCi per µmole) was supplied from New England Nuclear, Boston, USA.

*Preparation of LAHPO.* LAHPO was prepared from autoxidized LA by column chromatography and TLC as described by GAMAGE et al. (10). Purified LAHPO was characterized by POV (11) and UV absorption (12). In this preparation, LA and SP could not be detected by TLC.

*SP.* In the above column chromatography, SP was eluted by ethyl ether. The concentration of SP was expressed as acetaldehyde (13). The SP fractionated was free from LA and LAHPO when determined by TLC.

*FP.* The SP prepared was further aged at 37°C for 20 months. The little group of aldehyde could be detected in the SP aged. The aged SP contained short chain carboxylic acids, as generally recognized (14). The aged products were termed FP. The concentration of FP was expressed as acetic acid by titration with KOH.

*MA.* MA bis-(diethylacetal), (1, 1, 3, 3-tetraethoxypropane) was further purified and hydrolyzed as described by KWON and WATTS (15).
EFFECTS OF AUTOXIDIZED LINOLEIC ACID ON LYSOZYME

The molar absorptivity of MA was determined by UV absorbancy at 267 nm by using the molar absorptivity $\varepsilon = 3.18 \times 10^4$.

**Incubation of lysozyme with autoxidized products.** The incubation system consisted of 5 mg of lysozyme in 1 ml of 0.1 M of potassium phosphate buffer (pH 7.0) and 5 $\mu$moles of LA, LAHPO, SP, FP, BP, MA or PA. The mixtures in spitch glasses ($16.5 \times 105$ mm) were incubated at 37°C for 8 days, and control incubation was run in the autoxidized products-free system. The incubation mixture contained BP was preheated at 70°C for 1 hr.

**Removing methods of autoxidized products from incubation mixtures.** The mixtures incubated were washed several times with 2 ml of ethyl ether (Method I). Further, lysozyme was precipitated by addition of 4 ml of ethyl alcohol, centrifuged (3,800 rpm) and decanted. The lysozyme precipitated was macerated, ground and washed several times with 2 ml solution of ethyl ether: ethyl alcohol (1:1). The removing procedures involving Method I were referred to as Method II.

**Measurement of radioactivity.** LA-\textsuperscript{14}C ($6.92 \times 10^4$ cpm; the efficiency is 89%) was added into lysozyme solution. This mixture was incubated and washed as described above. An aliquot (0.1 ml) of the lysozyme solution was put on a filter paper disc (Whatman 3 MM), and dried. Radioactivity was measured with Packard Scintillation Spectrometer, Model 2425. The scintillant used consisted of 4 g of PPO and 0.1 g of POPOP in 1 liter solution of toluene: dioxane: ethylcellulose (1:1:1) containing 75 g of naphthalene.

**Amino acid analysis.** The washed lysozyme completely devoid of the washing solvents was hydrolyzed with 6 N HCl at 110°C for 23 hr. An aliquot of the hydrolysate equivalent to 1 mg lysozyme was analyzed with an amino acid analyzer, Hitachi Model KLA-3B. For the determination of tryptophan, lysozyme was hydrolyzed with 6 N HCl containing 3% purified thioglycollic acid as described by MATSUBARA and SASAKI (16). Through all processes, from the incubation to hydrolysis, the procedures were performed in an identical tube. The products of height and width (HW) of the eluted peaks of amino acids were calculated. The HW values obtained from the analyses of incubated lysozyme with autoxidized products were divided by those of control ($\times 100$, recovery %). One hundred percent minus the above recovery percentages were expressed as loss %. Free methionine, MetO$_1$ and MetO$_2$ were also determined quantitatively with the amino acid analyzer.

**Assay of lysozyme activity.** The lysozyme, incubated and treated with Method II, was dissolved in 5 ml of 0.1 M of potassium phosphate buffer (pH 7.1). One hundred microliters of lysozyme solution was added to a 3 ml suspension of *M. lysodeikticus* (0.5 mg) in 0.1 M phosphate buffer as described by SHUGAR (17). The decreasing rate of turbidity of suspension (at 450 nm) was compared with that of non-incubated lysozyme.

**Treatment with NaBH$_4$.** The precipitate of lysozyme incubated was suspended in 5 ml of water, and the pH was adjusted to 9.5 with NaOH. The suspension was
mixed with 10 mg of NaBH₄ and incubated overnight at 37°C (I8). The reaction was stopped by the addition of acetic acid to pH 5.

![Graph](image)

**Fig. 1.** Removal of the autoxidized LA-U-¹⁴C from the incubation system with lysozyme. The lysozyme incubated at 37°C for 1 hr (Δ–Δ) or 8 days (○–○) was washed as described in the text. From 1 to 5 is Method I, and from 1 to 8 is Method II. The lysozyme incubated for 8 days was treated with NaBH₄ after the washing with Method II, as described in the text.

**Table 1.** The losses of amino acids of lysozyme during the hydrolysis.

| Reactants          | LAHPO | LAHPO | LA   | LAHPO |
|--------------------|-------|-------|------|-------|
| Removal methoda    | I     | II    | II   | II    |
| Incubation temp. (°C) | 37    | 70    | 50   | 50    |
| Incubation time (hr) | 5     | 8     | 31   | 31    |

| Amino acids | Loss %b |
|-------------|---------|
| Lys         | 12      | 12     | 0    | 0     |
| His         | 24      | 5      | 3    | 3     |
| Arg         | 10      | 0      | 0    | 0     |
| Val         | 10      | 0      | 0    | 5     |
| Met         | 70      | 0      | 0    | 7     |
| Ileu        | 11      | 0      | 0    | 3     |
| Leu         | 9       | 0      | 3    | 4     |
| Tyr         | 10      | 0      | 0    | 0     |
| Phe         | 0       | 0      | 0    | 0     |

*a LA and LAHPO were removed from the incubation mixture by Method I or II as described in the text.

b The recoveries of the other kinds of amino acids were almost 100%, and the analysis of tryptophan was not done.
RESULTS AND DISCUSSION

Removal of autoxidized products from lysozyme incubation system

The method of removing autoxidized products from the lysozyme incubation mixtures was examined. The lysozyme was incubated with LA-\textsuperscript{14}C and washed as described in EXPERIMENTAL. The radioactivity remained in the system was measured (Fig. 1). LA-\textsuperscript{14}C was almost removed from the lysozyme solution, incubated for 1 hr, by washing three times with ethyl ether (Method I). However, in the system incubated for 8 days, 20\% of radioactivity still remained after the washing by Method I. When the lysozyme was precipitated by ethyl alcohol, the remaining LA-\textsuperscript{14}C decreased to 9\%. The precipitate was further washed several times with ethyl ether: ethyl alcohol (1:1), i.e., the lysozyme was treated with Method II. The residual autoxidized products in the lysozyme solution was finally 3\%. The different removing procedure, Methods I and II, showed a different result of the amino acid analysis as shown in Table 1. On lysozyme incubated with LAHPO at 37\textdegree C for 5 hr and washed by Method I, the loss of methionine was 70\%, and tyrosine, isoleucine, valine and basic amino acids were in the loss of 10\% or more. Even on lysozyme incubated with LAHPO at 70\textdegree C for 8 hr, when the lysozyme was treated with Method II, the amino acids were not lost except lysine. Many workers report that the autoxidized products form complexes with protein during incubation (19). These complexes could not be broken by extraction with ethyl ether, but were easily removed by precipitating lysozyme with ethyl alcohol. It seemed likely that these complexes which could not be removed by Method I were destroyed during the hydrolysis, and, consequently, the amino acids were lost on the analysis as shown in the leftmost column in Table 1. These results show that the amino acids of lysozyme were not damaged by LA and LAHPO under the mild condition such as incubation at 50\textdegree C for 31 hr. In the following experiments, Method II was used for removing the autoxidized products from the incubation mixture.

Lysozyme damage caused by the autoxidized products of LA

The effects of autoxidized products on the activity of lysozyme are shown in Fig. 2. Both SP and LAHPO remarkably inactivated lysozyme for the first 5 hr. LA decreased the activity up to 50\% after 40 hr. The effect of FP was the mildest among the products. Thus, the effects of these products were in the increasing order as follows: FP<LA<LAHPO<SP. However, the amino acid residues in lysozyme were not destroyed by these products in a short period incubation in which lysozyme was inactivated, as shown in Table 1. When lysozyme was incubated with LA and LAHPO at 45\textdegree C for 100 days, all amino acids were attacked at random by LA and LAHPO as shown in Table 2.

Therefore, the specific effects of autoxidized products on the amino acid residues in lysozyme were determined as shown in Table 3. On the comparisons of these effects of four groups of autoxidized products, the most suitable condition
Fig. 2. The effects on the activity of lysozyme by the autoxidized products. Lysozyme was incubated at 37°C with 5 μmoles of LA (○−○), LAHPO (●−●), SP (△−△) and FP (□−□), and washed with Method II as described in the text. Control reaction (×−×) was run in the autoxidized products-free system.

Table 2. Lysozyme amino acids damage by LA and LAHPO during the long incubation.

| Reactants | LA | LAHPO |
|-----------|----|-------|
| Amino acids | loss % |       |
| Lys       | 32 | 51    |
| His       | 43 | 50    |
| Arg       | 12 | 11    |
| Asp       | 5  | 18    |
| Thr       | 9  | 20    |
| Ser       | 0  | 16    |
| Glu       | 14 | 17    |
| Pro       | 24 | 13    |
| Gly       | 13 | 14    |
| Ala       | 12 | 13    |
| 1/2 Cys   | 14 | 15    |
| Val       | 14 | 14    |
| Met       | 18 | 19    |
| Ileu      | 14 | 27    |
| Leu       | 14 | 13    |
| Tyr       | 23 | 17    |
| Phe       | 12 | 13    |

* The mixture of lysozyme with LA or LAHPO were incubated at 45°C for 100 days.

examined was the incubation at 37°C for 8 days. The incubated lysozyme was treated with Method II and hydrolyzed for 23 hr in the identical tube to minimize the analytical loss. Only tryptophan, histidine and lysine residues were attrac-
Table 3. Lysozyme amino acids damage by autoxidized products of LA.

| Reactantsa | LA | LAHPO | SP | FP | BP | MA | PA | SP, NaBH₄b |
|------------|----|-------|----|----|----|----|----|------------|
| Amino acids | loss % | | | | | | | |
| Try        | 30 | 56    | 95 | 38 | 88 | 67 | 0  | 91         |
| Lys        | 11 | 17    | 73 | 7  | 21 | 36 | 5  | 76         |
| His        | 37 | 42    | 67 | 17 | 58 | 22 | 14 | 65         |
| Arg        | 7  | 9     | 14 | 11 | 23 | 11 | 0  | 18         |
| Asp        | 0  | 0     | 0  | 0  | 17 | 0  | 0  | 0          |
| Thr        | 5  | 5     | 9  | 0  | 17 | 18 | 11 | 9          |
| Ser        | 0  | 0     | 4  | 0  | 9  | 18 | 5  | 4          |
| Glu        | 4  | 4     | 17 | 0  | 17 | 6  | 14 | 8          |
| Pro        | 0  | —     | 0  | 0  | 35 | 8  | 9  | 11         |
| Gly        | 4  | 0     | 0  | 0  | 20 | 0  | 0  | 0          |
| Ala        | 5  | 0     | 0  | 0  | 21 | 0  | 0  | 0          |
| 1/2 Cys    | 4  | 0     | —  | 0  | 0  | 0  | 17 | 0          |
| Val        | 7  | 6     | 17 | 0  | 19 | 0  | 0  | 7          |
| Met        | 7  | 14    | 84 | 0  | 29 | 0  | 0  | 9          |
| Ileu       | 5  | 0     | 8  | 0  | 19 | 0  | 0  | 7          |
| Leu        | 0  | 0     | 5  | 3  | 21 | 0  | 0  | 6          |
| Tyr        | 5  | 0     | 19 | 5  | 38 | 0  | 0  | 10         |
| Phe        | 0  | 0     | 6  | 0  | 24 | 0  | 0  | 0          |

a Experimental conditions shown in text.

b The lysozyme incubated with SP was treated with NaBH₄ as described in the text.

tively lost by autoxidized products. LA damage was up to 30% of tryptophan and 40% of histidine. LAHPO damage was up to 50% of tryptophan and histidine. SP destroyed almost all tryptophan, and 70% of histidine. Besides, SP damage was up to 80% of methionine and lysine, and to 20% of tyrosine, valine and glutamic acid, unlike the other autoxidized products. FP affected only on tryptophan. Thus, SP was the most destructive, and FP was the mildest reactant among these products. It is well known that both LA and LAHPO are decomposed and produced SP during incubations. The longer incubation time, the more SP was produced from LA and LAHPO. But FP was not further oxidized. These results and those in Tables 1 and 2 show that the amino acid residues in lysozyme were specifically destroyed by SP.

The effects of standard reagents on the amino acid residues in lysozyme were compared with those of autoxidized products. BP, MA and PA were used as the standard reagents of a radical reagent, aldehyde compound and short chain carboxylic acid, respectively (20). The effect of BP was drastic. Tryptophan (88%), histidine (58%), proline (35%) and tyrosine (38%), which were heterocyclic or unsaturated aromatic compounds and were radical trappers (21), were remarkably lost by the attack of benzoyl radical. The effect of BP on the amino acid residues was not similar to those of LA and LAHPO, while the degradation processes of LA and LAHPO were radical reactions (22). MA was not so destructive as SP,
but affected threonine and serine (18%). The effect of MA was different from that of SP, while SP contained appreciable amounts of aldehyde compounds (23). Tryptophan was not destroyed by PA unlike FP, while FP was, for the most part, composed of short chain carboxylic acid (14). Thus, the effects of standard reagents were not similar to those of autoxidized products. The autoxidized products might have a stimulative effect on the destructions of amino acid residues, besides the action of each functional group, –OOH, –CHO and –COOH.

**Effect of SP on the methionine residue in lysozyme**

The most interesting change is shown on the line of methionine in Table 3. Methionine residue in lysozyme was specifically lost by SP. When lysozyme incubated with SP was treated with NaBH₄ as described in EXPERIMENTAL, the 84% loss of methionine residue became 9%. Therefore, free methionine to equimolar with the methionine residue in the lysozyme system was incubated with SP and was treated under the same condition as the lysozyme system (Table 4). The methionine changed to MetO₁, MetO₂ altered to MetO₂, and MetO₂ was not further oxidized by SP. Both MetO₁ and MetO₂ were not reduced to methionine by NaBH₄. On the other hand, when the lysozyme was hydrolyzed in the system contaminating SP, only 30% of methionine was lost. MetO₁ and MetO₂ were reduced under the hydrolysis condition rather than were oxidized, even in the system containing SP. As shown in Fig. 1, the trace amounts of autoxidized products still remained in the

| Samples | Amounts (µmole) | Treatmentsᵃ | Products (µmole) | MetO₁ | MetO₂ | Met |
|---------|----------------|-------------|----------------|-------|-------|-----|
| Met     | 0.521          | —           | NDᵇ           | ND    | ND    | 0.520 |
| MetO₁   | 0.654          | —           | 0.608         | ND    | 0.046 |
| MetO₂   | 0.680          | —           | 0.036         | 0.645 | ND    |
| Met     | 0.521          | with SP for 8 days | 0.342 | ND    | ND    |
| MetO₁   | 0.654          | with SP for 8 days | ND    | 0.543 | ND    |
| MetO₂   | 0.680          | with SP for 8 days | ND    | 0.467 | ND    |
| MetO₁   | 0.654          | treated with NaBH₄ | 0.612 | ND    | 0.050 |
| MetO₂   | 0.680          | treated with NaBH₄ | 0.032 | 0.663 | ND    |
| Met     | 0.521          | heated with SPᵉ | ND            | trace | 0.519 |
| MetO₁   | 0.654          | heated with SP   | 0.062         | 0.023 | 0.448 |
| MetO₂   | 0.680          | heated with SP   | 0.710         | ND    | 0.013 |
| Met in lysoyme | 0.702 | hydrolyzed with SPᵈ | ND | ND | 0.484 |
| Met in lysoyme | 0.702 | hydrolyzed with reduced SPᵉ | ND | ND | 0.498 |

ᵃ Experimental conditions same as in Table 3.
ᵇ ND, abbreviation for non-detectable.
ᵉ Samples were heated at 110°C for 23 hr in system containing 5×10⁻⁴ M of SP as acetaldehyde.
ᵈ The lysozyme was hydrolyzed in system containing 5×10⁻⁴ M of SP as acetaldehyde, and other kinds of amino acids were not almost lost.
ᵉ SP (5×10⁻⁸ M as acetaldehyde) was reduced by NaBH₄ to 0.3×10⁻⁸ M.
lysozyme, even after washing by Method II. The remaining 250 cpm of LA–U–14C decreased to 80 cpm by the treatment with NaBH₄. As clarified from discussion in Table 1, the methionine residue in lysozyme formed the complex with SP during the incubation. It was easy to remove SP from the incubation mixture of free methionine, but it was difficult to break the complex between methionine residue in lysozyme and SP by Method II, as described by TAPPEL (24). But the complex was broken by hydrogen generated from the decomposing NaBH₄. This effect of NaBH₄ might protect the destruction of methionine residue during the hydrolysis of lysozyme. Thus, it was considered that the methionine residue was not lost in the system containing SP during the hydrolysis, but the complex of methionine residue with SP was specifically destroyed during acid-hydrolysis. Actually, no methionine residue was easily lost by autoxidized products.

The lysozymes incubated with the other reactants than SP were also treated with NaBH₄. No recovery of the lost amino acids was observed, as well as the losses of tryptophan, lysine and histidine by SP were not recovered by the treatment with NaBH₄ as shown in the rightmost column in Table 3. Though the autoxidized LA–U–14C could be almost completely removed from the lysozyme system by the treatment with NaBH₄ (Fig. 1), unlike the lost amino acids were not recovered methionine. Thus, the losses of these amino acids did not occur during acid-hydrolysis, unlike the case of methionine. Tryptophan, lysine and histidine were destroyed directly by the attacks of autoxidized products during the incubations.

On the bases of these results, it was concluded that the susceptible tryptophan, lysine and histidine residues in lysozyme were specifically damaged by SP. Studies on the reaction products of amino acid residues with SP must be done, and a preventable way of the toxicity of SP must also be examined (25).

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