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Oxidative Degradation *in vitro* of a Receptor Lipopolysaccharide for Bacteriophage ϕX174, by Low-molecular-weight Amadori Rearrangement Product

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Abstract

Oxidative degradation of a lipopolysaccharide (LPS) by a low-molecular-weight Amadori rearrangement product was studied as an *in vitro* model for glycated protein-mediated degradation of biopolymers under physiological conditions. LPS isolated from *Escherichia coli* C (*E. coli* C) exhibited *in vitro* inactivating activity against bacteriophage ϕX174. The reaction of LPS (2 μg/ml) with an arylamine Amadori rearrangement product (0.5 mM) was examined in a phosphate buffer at pH 7.4 for 6 h in the presence of Cu^{2+} (10^{-5} M). The results showed that the inactivation reaction by LPS (2 μg/ml) was inhibited by 40%, showing that LPS lost its inactivating ability against the phage *in vitro*. The degradation of LPS was dependent on the concentration of the Amadori rearrangement product and the reaction time of mixing LPS with the Amadori rearrangement product. Oxygen radical scavengers inhibited this reaction, demonstrating that LPS was oxidatively damaged by oxygen radical species generated by the Amadori rearrangement product.

Key words : lipopolysaccharide • oxidative degradation • Amadori rearrangement product • inactivation of ϕX174

1. Introduction

DNA, protein, polyunsaturated fatty acid and carbohydrate are well-known as the target molecules in the field of active oxygen radical research. Structural damages to these biomolecules such as chemical modification of amino acid residues, scission of DNA, cleavage of carbohydrate chain, lead to their biofunctional loss, as shown in a number of the past studies. In contrast, the effects of active oxygen radicals on more complex macromolecules, glycoconjugates, such as glycoproteins, glycolipids, lipopolysaccharides have received less attention and information about these reactions is very limited. Structural damages to gangliosides[1] and a sialyloligosaccharide[2] by hydroxyl radical have been reported recently. These macromolecules exist in outer layers of cells and may be targets of active oxygen species formed in living systems, and radical-mediated damage to these macromolecules,

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hence, might be involved in oxidant-induced alteration of cellular functions.

Lipopolysaccharides exist in bacterial outer membranes and some of them are well known as the receptors for the bacteriophage ϕX174. *E. coli* C and *S. typhimurium* are its host bacteria.[3] The interaction of the phage with LPS has received much attention for its interesting molecular functions of recognition and of transferring genes into the host cells by the virus proteins and LPS molecules.

In our laboratory, in order to know the mechanism interaction of the bacteriophage with *E. coli* C on a molecular level, researches on the synthesis of oligosaccharides of LPS, their biological activity, and analysis of their conformation have been performed.

During the course of our study on the autoxidation of reducing sugars, it was reported that the Amadori rearrangement products, the primary intermediates in the Maillard reaction, generate active oxygen species[4] and these species cause oxidative cleavage of DNA, inactivation of viruses[5] and oxidative depolymerization of hyaluronic acid.[6] In this study, LPS was chosen as another target macromolecule for active oxygen radicals formed from the Amadori rearrangement products. We describe here the reaction of LPS with the Amadori rearrangement product. The LPS was exposed to the autoxidation of the Amadori rearrangement product. The effect on the biological activity of LPS was then examined by the method of plaque assay for determination of LPS receptor activity. Intact LPS inactivates ϕX174 *in vitro*, whereas structurally damaged LPS is expected to lose its inactivating activity.

2. Materials and Methods

Materials

A low-molecular-weight Amadori rearrangement product, 1-deoxy-1-*p*-toluidino-D-fructose was prepared as described before.[4] Yeast extract, D-glucose, agar, sodium chloride, calcium chloride, potassium chloride, magnesium sulfate, tris (hydroxymethyl) aminomethane (Tris) and other chemicals were obtained from Nakalai Tesque Co. and were of guaranteed reagent. Polypepton was obtained from Wako Pure Chemical Industries Ltd. Redistilled water was used throughout the experiments.

Phage and Bacterium

Bacteriophage ϕX174 (wild type), was originally obtained from Dr. T. Komano, Department of Agricultural Chemistry, Kyoto University, Japan. It was grown and purified by the method of a literature[7] in this laboratory by H. Kojima. *E. coli* C, was also obtained originally from Dr. T. Komano. It was cultivated in a culture medium (polypepton: 1%, sodium chloride: 0.5%, extract yeast: 0.5%, glucose: 0.1%, 1 M of calcium chloride: 0.01%). After harvesting, *E. coli* C was centrifuged for 10 min at 7000 rpm (4℃) and the solution of *E. coli* C in 0.01 M Tris-HCl buffer (OD_{600}=0.5) was prepared for plaque assay. A refrigerated centrifuge of Tomy Seiko Co. Ltd. (RB-18IV, 9000 rpm) and an autoclave of Alupu Co. (KT 30L) were used in this experiment.

Isolation and Purification of a Lipopolysaccharide from E.coli C

A lipopolysaccharide was extracted with a mixture containing liquid phenol (90 g dry phenol+11 ml water), chloroform and petroleum ether in a volume ratio of 2 : 5 : 8, from the acetone powder of *E.*

coli C bodies and purified by the method of literature.[8]

Reaction of LPS with the Amadori Rearrangement Product

LPS (1 or 2 μg/ml) was incubated with 1-deoxy-1-*p*-toluidino-D-fructose (0.05 ~ 0.5 mM) and Cu^{2+} (10^{-5}M) in 0.01 M Tris-HCl buffer at 37℃ for 6 h. Sterilized water instead of a solution of the Amadori rearrangement product and Cu^{2+} was incubated with LPS, as the control run. After the reaction, the phage receptor activity of LPS was examined by the plaque assay. To avoid the reaction of excess of the Amadori rearrangement product with ϕX174, two methods were used in this experiment. After the reaction, the reaction mixtures were dialyzed in deionized water for two days to remove the excess of the Amadori rearrangement product and other low-molecular-weight compounds. Alternatively, after the reaction tiron (10 mM) was added to the reaction mixture to stop the reaction.

Plaque Assay (Estimation of Receptor Activity of LPS)

The typical plaque assay was used to examine phage receptor activity of LPS is lost after the treatment with the Amadori rearrangement product. The phage receptor activity of LPS was measured as a survival ratio of ϕX174 after mixing the phage with intact LPS or LPS treated with the Amadori rearrangement product. Ten μl of ϕX174 (8.26×10^8 plaque-forming units/ml) and 100 μl of the solution of intact LPS or treated LPS (2 μg/ml) were added to 0.01 M Tris-HCl buffer (pH 8.1), containing 5g of KCl, 1g of NaCl, 0.2g of $MgSO_4$ and 1 mM $CaCl_2$ per L. The reaction mixture (1.0 ml) was incubated at 37℃. After incubation of 0 h, 1 h, 2 h and 3 h, aliquots were withdrawn, diluted 10000-fold with the same buffer, and this diluted solution of 100 μl was added to 300 μl of *E. coli* C ($OD_{600}=0.5$), prewarmed at 37 ℃, and 5ml of top agar, and assayed for plaque-forming ability on *E. coli* C by the agar overlayer method of Adams.[9] All the experiments were performed in triplicates. After incubation of agar plates for 3 h at 37℃, the numbers of plaque (A) were counted in dilutions: 10-200 plaques/100 μl, A is between $A-2\sqrt{A} \leqq$ and $\leqq A+2\sqrt{A}$. The phage receptor activity of LPS was expressed as a survival ratio (%). It was calculated by dividing of the number of plaque-forming units after 1 h, 2 h or 3 h treatment with LPS by the number of plaque-forming units of 0 h control which was spread on a agar plate immediately after addition of a LPS solution.

Inhibition of the Oxidative Degradation of LPS by Scavengers of Active Oxygen Radicals

Scavengers were added to the reaction of LPS with the Amadori rearrangement product and Cu^{2+}. The inhibition ratio was expressed as Pt-P/Pt-Pl$\times$100%. Pt is the survival ratio of ϕ X174 treated with LPS which was incubated with the Amadori rearrangement product and Cu^{2+}. Pl is the survival ratio of ϕ X174 treated with LPS. P is the survival ratio of ϕ X174 treated with LPS mixture which was incubated with the scavengers, and the Amadori rearrangement product, and Cu^{2+}.

3. Results and Discussion

Inactivation of Bacteriophage by LPS Isolated from E. coli C

The bacteriophage ϕX174 is inactivated *in vitro* by lipopolysaccharide isolated from its host *E. coli* C

so that no longer form plaques *in vivo on* lawns of the host organism. At the same time, the viral DNA becomes susceptible to deoxyribonuclease (eclipse), as it does at the beginning of infection to the host bacterium. As shown in Fig. 1A, after incubation at 37℃ for 3 h, the survival ratio of ϕX174 of 8.26×10^8 p.f.u./ml decreased only to 91%, but decreased to 0.7% when 2 μg/ml of LPS was added. In the presence of LPS, 99.3% of ϕX174 was killed and the result showed that the isolated LPS has a desired ϕX174 inactivating activity.

The concentration of LPS needed to cause the phage inactivation is shown in Fig. 1B. Whereas below 10^{-2} μg/ml of LPS, inactivation of ϕX174 was not observed, between 0.01 ~ 0.1 μg/ml, some inactivation was observed. At concentration of 1.25 μg/ml survival ratio was found to be 10% and at concentration of 2 μg/ml, survival ratio was found to be 0.7%. So in this experiment, we chose 2 μg/ml as a LPS concentration, at which the degree of inactivation of ϕX174 was found to be appropriate for use in determining the effect of active oxygen radical on LPS.

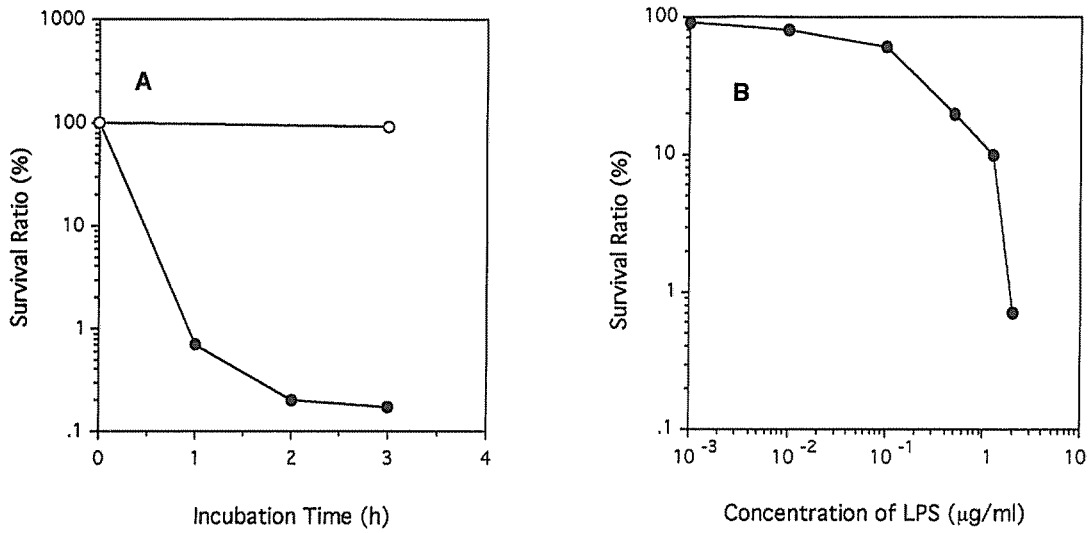

Fig. 1 Inactivation of ϕX174 by LPS.

A: ϕX174 (8.26×10^8 p. f. u. /ml) was incubated without (○) or with (●) 2 μg/ml of LPS in 0.01 M Tris-HCl buffer (pH 8.1) for 3 h at 37℃. An aliquat was removed, diluted 10^4-fold with the same buffer and assayed for infectious phage by the agar over-layer method of Adams.

B: The Effect of LPS Concentration on Inactivation of ϕX174. ϕX174 (8.26×10^8 p. f. u. /ml) was incubated with LPS in 0.01 M Tris-HCl buffer (pH 8.1) for 1 h at 37℃.

Degradation of the LPS by the Low-molecular-weight Amadori Rearrangement Product

As described in Methods and Materials, LPS (2 μg/ml), and 1-deoxy-1-*p*-toluidino-D-fructose (10^{-4}M) in the presence of Cu^{2+} (10^{-5}M) in 0.01 M Tris-HCl buffer (pH 8.1) was incubated at 37℃ for 6 h. After incubation, 10 mM tiron was added to stop the reaction. Then the phage-receptor activity of LPS solution treated with the Amadori rearrangement product was assayed by plaque assay. We previously reported

that inactivation of ϕX174 by the Amadori rearrangement product is due to strand scission of viral DNA. So we firstly examined whether the concentration of the Amadori rearrangement product used in this experiment had any effect on ϕX174. The result showed that the Amadori rearrangement product caused 50% inactivation of ϕX174 in the absence of LPS. But 10 mM of tiron could inhibit this effect as shown in Fig. 2. Blank experiment showed that tiron alone had no effect on inactivation of ϕX174. It can be concluded that excess amount of the Amadori rearrangement product has no effect on ϕX174 after addition of 10 mM of tiron. So the LPS reaction mixture after treatment with the Amadori rearrangement product was used directly to react with ϕX174 and plaque assay. In Fig. 2, after incubation for 3 h, the survival ratio of ϕX174 was found to be 0.17% when LPS was added. On the other hand, the survival ratio of ϕX174 increased to 40% by LPS which treated with the Amadori rearrangement product and Cu^{2+}. This means that the LPS lost its receptor activity after treatment with the Amadori rearrangement product. It is very possible that the active oxygen radicals generated from the Amadori rearrangement product cause the degradation of LPS. In this experiment, however, no further structural study on the reacted LPS was made.

Effect of Reaction Time and Concentration of the Amadori Rearrangement Product on the Oxidative Degradation of LPS

The relationship between reaction time of LPS with 0.2 mM of 1-deoxy-1-*p*-toluidino-D-fructose in the presence of Cu^{2+} (10^{-5}M) and the survival ratio of ϕX174 is shown in Fig. 3A. The survival ratio of ϕX174 increased greatly within 6 h's incubation time when LPS was incubated with the Amadori rearrangement product.

The degradation of LPS was dependent on the concentration of 1-deoxy-1-*p*-toluidino-D-fructose, as shown in Fig. 3B. The survival ratio of phage increased when the concentration of 1-deoxy-1-*p*-toluidino-D-fructose ($0 \sim 2\times10^{-4}$ M) increased. When the concentration of 1-deoxy-1-*p*-toluidino-D-fructose was up to 2×10^{-4} M, the survival ratio of ϕX174 decreased greatly, because ϕX174 was also damaged by the Amadori rearrangement product (data not shown).

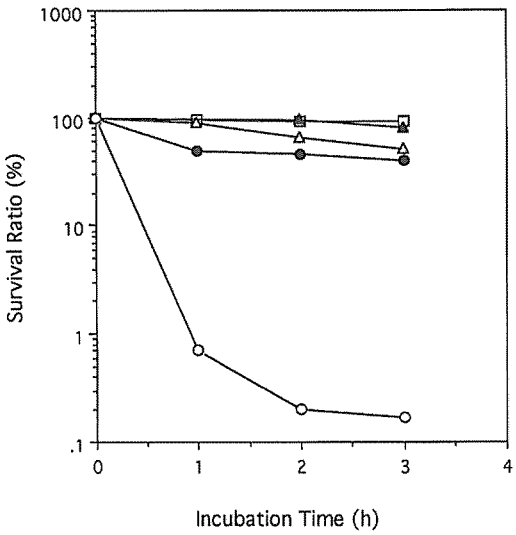

Fig. 2 Degradation of LPS by the Amadori Rearrangement Product.

LPS reaction mixtures containing LPS (2 μg/ml), 1-deoxy-1-*p*-toluidino-D-fructose (10^{-4} M) in the presence of Cu^{2+} (10^{-5}M) in 0.01 M Tris-HCl buffer (pH 8.1) were incubated in 37℃ for 6 h. After addition of 10 mM of tiron, the LPS reaction mixture was incubated with ϕX174 (8.26×10^{8} p.f.u./ml), diluted with buffer and assayed for plaque-forming ability on *E. coli* C by the agar over-layer method of Adams, as described in Materials and Methods.
ϕX174:□; ϕX174+Tol+Cu^{2+}:△; ϕX174+Tol+Cu^{2+}+Tiron:▲; ϕX174+LPS:○; ϕX174+LPS+Tol+Cu^{2+}+Tiron: ●.

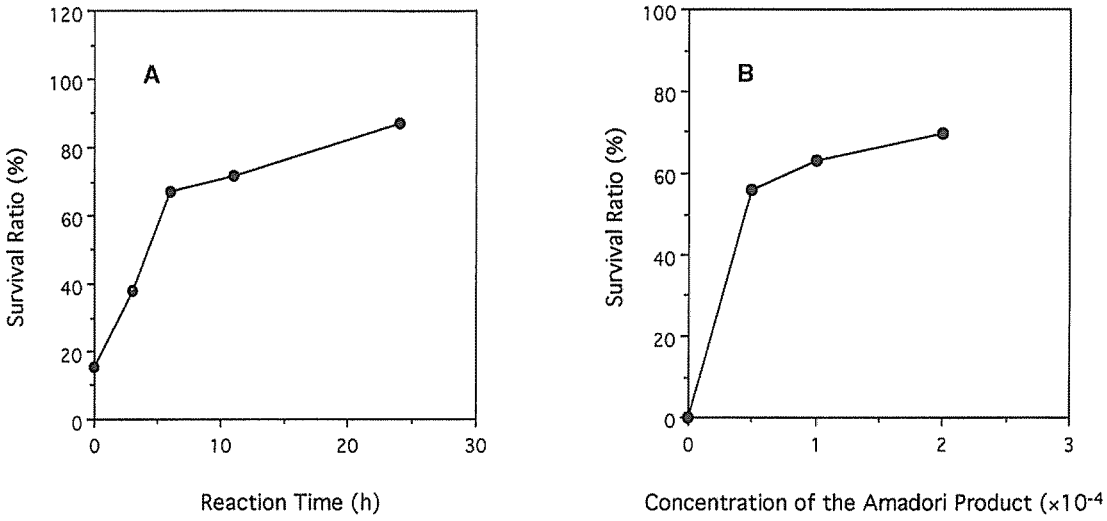

Fig. 3 The Effects of Reaction Time and Concentration of the Amadori Rearrangement Product on Loss of Receptor Activity of LPS for Inactivation of ϕX174.

A: The Effect of Reaction Time of Mixing LPS with Amadori Rearrangement Product. LPS reaction mixtures containing LPS (2μg/ml), 1-deoxy-1-*p*-toluidino-D-fructose (2×10^{-4}M) in the presence of Cu^{2+} (10^{-5}M) in 0.01 M Tris-HCl buffer (pH 8.1) were incubated in 37℃ and assayed by the plaque assay, as described in Fig. 2.

B: The Effect of Concentration of the Amadori Rearrangement Product. The reaction condition is same with that as described above.

Effect of Active Oxygen Radical Scavengers on the Degradation

To confirm whether the degradation of LPS was induced by oxygen radicals generated from the Amadori rearrangement product, the effects of various agents and enzymes which scavenge oxygen radicals were examined. Superoxide dismutase, tiron, and scavengers for superoxide radical inhibited the LPS degradation (Table). Some nonspecific scavengers for hydroxyl radical such as mannitol, thiourea, and DMSO inhibited partially the LPS degradation. Effective inhibition by DETAPAC, catalase, suggests the roles of metal ion and hydrogen peroxide. These results indicated that some, or all, of these oxygen-derived radicals are involved in the observed degradation of LPS.

Proteins, DNA, polyunsaturated fatty acids and carbohydrates have long been studied by various systems of oxygen radicals. The effects of oxygen radicals on glycoconjugates have received somewhat less attention. Recently several studies of oxidative damage to ganglioside have been reported. But there has been few report about oxidative damage on lipopolysaccharide. The results of the present study show that under the physiologic condition LPS was oxidatively degraded by autoxidizable Amadori rearrangement product, leading to loss of its receptor activity for ϕX174. The autoxidation of the Amadori rearrangement product was reported previously and active oxygen radicals were found to be generated from the Amadori rearrangement products. Some features of the degradation of LPS by autoxidizable Amadori rearrangement product are very similar to those of phage inactivation, HA

Table The Effects of Oxygen Radical Scavengers and Metal Chelators on the Inactivation of LPS, A Receptor for Bacteriophages ϕ X174, by A Amadori Rearrangement Product in the Presence of Cu^{2+}.

Additive	Concentration (mM)	Inhibition (%)
SOD	110 U/ml	83.0
Catalase	280 U/ml	80.6
Tiron	1	73.7
DETAPAC	0.1	93.5
Mannitol	50	15.7
Thiourea	10	57.0
DMSO	100	50.7

LPS reaction mixtures containing LPS (2 μg/ml), 1-deoxy-1-*p*-toluidino-D-fructose (10^{-4}M) in the presence of Cu^{2+} (10^{-5}M) and scavengers in 0.01 M Tris-HCl buffer (pH 8.1) were incubated at 37℃ for 6 h. After addition of 10 mM of tiron, the LPS reaction mixture was incubated with ϕ X174 (8.26×10^{8} p.f.u./ml), diluted with buffer and assayed for plaque-forming ability on *E. coli* C by the agar over-layer method of Adams, as described in Materials and Methods. Inhibition ratio was expressed as Pt-P/Pt-Pl$\times$100%. Pt is the survival ratio of ϕ X174 reacting with LPS reaction mixture which was incubated with the Amadori rearrangement product and Cu^{2+}. Pl is the survival ratio of ϕ X174 reacting with LPS. P is the survival ratio of ϕ X174 reacting with LPS reaction mixture which was incubated with the scavengers, Amadori rearrangement product and Cu^{2+}.

depolymerization and nucleic acids strand scission by Amadori rearrangement products, reducing sugar phosphates and reductones such as ascorbic acid. Qi and Kashimura reported that fructose 6-phosphate also gave rise to similar damage to LPS.[10]

The observed inhibitory effects of SOD, catalase and other radical scavengers suggest that superoxide, hydrogen peroxide and hydroxyl radical are involved in the LPS degradation. Superoxide radical is known to (a) undergo dismutation to yield hydrogen peroxide, and (b) react with hydrogen peroxide to generate hydroxyl radical. Hydroxyl radical is suggested as responsible species for HA, DNA and protein degradation with many autoxidizable compounds. Some scavengers for hydroxyl radical, however inhibited partially the degradation of LPS by the Amadori rearrangement product.

Phage inactivation, DNA strand scission and depolymerization of HA by the Amadori rearrangement products are stimulated by Cu^{2+}. In this work, we have not examined whether the degradation of LPS can be stimulated by Cu^{2+} alone and whether a complex between Cu^{2+} and LPS is formed or not. But the effective inhibitory effect by metal chelate of DETAPAC suggested that trace amounts of Cu^{2+} in the reaction mixture may be involved in autoxidation of the Amadori rearrangement product. The degradation of LPS may be due to the Fenton-like reaction. Although redistilled water was used throughout the experiments, Cu^{2+} is known to have a catalytic effect on autoxidation.[11] It was reported that polysaccharides containing phosphoester bond can effectively condense trace metal ions such as Cu^{2+}.[12] As the present lipopolysaccharide have phosphoester bonds, they may have a similar function of trace metal ions condensation.

The other features of degradation of LPS by the Amadori rearrangement product are still not clear on this stage. It remains to be studied either sugar residues or lipid part of LPS are preferablely cleaved.

The position of scission among sugar residues is thought to be important in the degradation of LPS, since neutral sugar chain play an important role in the recognition of the phage.[13]

In the present study, we have described the degradation of a LPS, receptor for ϕX174, by the Amadori rearrangement product in the presence of copper ion. The present work has demonstrated that LPS is damaged by oxygen radical generated from the Amadori rearrangement product and loses its receptor activity for ϕX174 *in vitro*. The loss of the receptor activity was dependent on the concentration of the Amadori rearrangement product used and reaction time of LPS with the Amadori rearrangement product. Oxygen radicals scavengers inhibited this reaction, suggesting that LPS was oxidatively damaged by the Amadori rearrangement product. But LPS damages portion is not clear and remains to be studied. LPS exists in bacterial outer membrane. Membranes are very susceptible to peroxidation by active oxygen species.[14] LPS may be a target of active oxygen species and this oxidative damage to LPS may take place in vivo.

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References

1) KONAGAYA, N., M.INAGAKI, S.NISHIKAWA, and N.KASHIMURA. Facile reaction of gangliosides with hydroxyl radical, *Redox Report*, 2 (3): 185−189 (1996).

2) KOKETSU, M., R.SUZUKI, S. NISHIKAWA, and N. KASHIMURA. *In vitro* degradation of a sialyl-oligosaccharide derivative by hydroxyl radical, *Carbohydr. Lett.*, 1: 199−206 (1995).

3) WILKINSON, S. G. Composition and structure of bacterial lipopolysaccharides in *Surface Carbohydrates of the Prokaryotic Cell* (ed. by I. Sutherland, Academic Press, London), p97−176 (1977).

4) ZU, J., J.MORITA, S.NISHIKAWA, and N.KASHIMURA. Generation of active oxygen species by some low-molecular Amadori rearrangement products under physiological conditions, *Carbohydr. Lett.*, 1: 457−464 (1996).

5) KASHIMURA, N., J.MORITA, K.SUGIURA, S.NISHIKAWA, and Z.KUMAZAWA. Autoxidation and *in vitro* DNA-cleavage activity of some nitrogenous derivatives of reducing sugars in *The Role of Oxygen in Chemistry and Biochemistry* (ed. by W. Ando, Y. Moro-Oka, ED; Elsevier, Amsterdam), 33: p483−488 (1988).

6) ZU, J., S.NISHIKAWA, and N.KASHIMURA. Depolymerization of hyaluronic acid by low-molecular-weight Amadori-rearrangement products and glycated polylysine, *Biosci. Biotech. Biochem.*, 61 (1): 188−190 (1997).

7) KOMANO, T. ϕ X174 Bacteriophage, *Protein, Nucleic acid and Enzyme*, 9(2): 26 − 30 (1973) (in Japanese).

8) GALANOS, C., O.LUDERITZ, and O.WESTPHAL. A new method for extraction of R lipopolysaccharides, *Eur. J. Biochem.*, 9(2): 245−249 (1969).

9) ADAMS, M. H. Bacteriophages, Interscience Publisher, New York, p450−454 (1959).

10) QI, L. Thesis of Master, Mie University, 1994.

11) BARRON, E. S. G., Z. B.MILLER, and G.KALNITSKY. Oxidation of dithiols, *Biochem. J.*, 41: 62–68 (1947).

12) SAKAGUCHI, T., T.HORIKOSHI, and A.NAKAJIMA. Studies on the accumulation of heavy metal elements in biological systems, part VII. Adsorption of heavy metal ions by chitin phosphate and chitosanphosphate, *Nippon Nogei Kagaku Kaishi*, 53(5): 149–56 (1979) (in Japanese).

13) FEIGE, U. and S.STIRM. On the structure of the *Escherichia coli* C cell wall lipopolysaccharide core and on its ϕX174 receptor region, *Biochem. Biophys. Res. Commun.*, 71(2): 566–573 (1976).

14) YAMAMOTO, Y., E.NIKI, J.EGUCHI, Y.KAMIYA, and H.SHIMASAKI. Oxidation of biological membranes and its inhibition. Free radical chain oxidation of erythrocyte ghost membranes by oxygen, *Biochim. Biophys. Acta*, 819: 29–36 (1985).

アマドリ転位化合物によるバクテリオファージφX174 レセプター活性を有するリポ多糖（LPS）の酸化的損傷反応

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グリケート化タンパク質のモデル化合物として，低分子アマドリ転位化合物によるリポ多糖（LPS）の酸化損傷反応を検討した．大腸菌C株から得られたバクテリオファージφX174のレセプターとしての生理活性を有するＬＰＳは，プラーク検定法で，その *in vitro* でのファージ不活化活性を調べたところ，温度37℃で，アマドリ化合物による６ｈの処理で，LPSのファージ不活化活性が失われることが認められた．また，この反応はアマドリ転位化合物の濃度と反応時間に依存し，活性酸素ラジカル阻害剤などによって阻害されることから，アマドリ転位化合物から生成する活性酸素は生理的条件下で，生理活性を有するリポ多糖の機能的，構造的な損傷を引き起こしたことを示した．

この結果は，*in vitro* でアマドリ転位化合物が生成すれば，LPSの機能的な損傷が起こり得ることを示している．