Coexpression of Trypsin Inhibitory and Acceleratory Substances during Autoxidation of Methyl Linolenate : Confirmation of Trypsin Inhibitory Substances

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Abstract : During the autoxidation of methyl linolenate, a group of degradation products capable of inhibiting β -trypsin activity were generated along with the accelerating substances, as also noted in a previous study. Chemical modification of the functional groups indicated the carbonyl and hydroxyl groups of the inhibitory degradation products and ϵ -amino group(s) of trypsin to possibly be essential for the inhibition, but hydroperoxide not necessarily involved in inhibitory activity. The inhibitory degradation products inhibited α -chymotrypsin and lysozyme activity and thus may be inhibitors of various enzymes. There is thus the possibility that enzyme inhibitors and accelerators are generated spontaneously during the autoxidation of unsaturated lipids.

Key words : autoxidation, methyl linolenate, trypsin, chymotrypsin, lysozyme, inhibitor, accelerator

1 Introduction

Lipid oxidation is now recognized to be a critically important reaction in physiological processes and in food products. Toxicological researches about lipid oxidation products have been carried out both *in vivo* and *in vitro* systems, and lipid oxidation products such as lipid hydroperoxides and their secondary degradation products including 4-hydroxynonenal^{(1)~(3)} were suggested to repress enzymatic activity^{(4)~(9)}. We have been studied *in vitro* effect of oxidized unsaturated lipids on enzymes, mainly on bovine pancreatic β -trypsin^{(10)~(12)}, which is advantageous for these assessments because of the well-elucidated properties such as substrate specificity, chemical structure, and inhibitors. In the previous paper⁽¹¹⁾, we reported that a group of degradation

products obtained from autoxidized methyl linolenate (oxd MLn) could significantly enhance the amidase activity of β -trypsin and, at the same time, some other degradation products appeared to depress that activity. In this study, we confirmed the coexistence of these inhibitory substances with the acceleratory ones in the degradation products of oxd MLn.

2 Materials and Methods

2.1 Separation of Active Fractions from Autoxidized Methyl Linolenate

Four grams of MLn (Tokyo Kasei Kogyo) was oxidized for 48 h at 40°C in the dark with a gentle-stirring. The resulting oxd MLn (Peroxide value Ca. 2400 meq/kg) was separated by gel chromatography on a Sephadex LH-20 column (2.5×120 cm) using acetone as the eluent at a flow rate of 1.0 ml/min. The active fractions obtained were partially purified by a prepacked silicic acid

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chromatography (LiChroprep Si-60, 2×31 cm, E. Merk) using *n*-hexane and 2-propanol (95/5, vol/vol) as the eluent at a flow rate of 0.5 ml/min¹¹. The active fractions obtained were allowed to react with each of enzymes under the suitable conditions and the residual enzyme activity was measured.

2.2 Assay of Enzymatic Activity

β -Trypsin was isolated from bovine pancreatic trypsin (Sigma Chemical Co., 3× crystallized) by the method of Shroeder and Shaw¹³. ϵ -*N*-Acetylated trypsin (Sigma Chemical Co.), bovine pancreatic α -chymotrypsin (Sigma Chemical Co., 3× crystallized, dialyzed and lyophilized), and chicken egg white lysozyme (Sigma Chemical Co., 3× crystallized, dialyzed and lyophilized) were used as received.

After the reaction of each enzyme with oxd MLn, the residual enzymatic activity was assayed as follows. Amidase activity of β -trypsin was measured using α -*N*-benzoyl-DL-arginine-*p*-nitroanilide·HCl (BAPNA, Sigma Chemical Co.) as substrate¹⁰. In some cases, α -*N*-*p*-tosyl-L-arginine methyl ester (Sigma Chemical Co.) and milk casein (Wako Pure Chemical Ind., Ltd.) were used as the esterase and proteolytic substrates of β -trypsin¹². Enzymatic activity of α -chymotrypsin⁷ and lysozyme⁶ were each measured using milk casein and cells of *Micrococcus lysodeikticus* ATCC No.4698 (Sigma Chemical Co.) as substrates.

2.3 Chemical Modification of Functional Groups of Active Degradation Products

Functional groups of the active degradation products obtained from oxd MLn were chemically modified by the method of Nakamura et al^{12,14,15}. The active degradation products containing carbonyl and hydroxyl groups were converted to dimethylhydrazone (DMH) derivatives using *N*, *N*-dimethylhydrazine (Nacalai Tesque, Inc.) and trimethylsilyl (TMS) ether derivatives using hexamethyldisilazane and trimethylchlorosilane (Nacalai Tesque, Inc.), respectively^{12,14}. Hydroperoxide(s) was reduced to hydroxyl group(s) using triphenylphosphine (TP, Wako Pure Chemical Ind., Ltd.)¹⁵.

3 Results

3.1 Separation of Active Degradation Products from oxd MLn

Figure 1 shows separation of oxd MLn and

Fig. 1 Separation of Autoxidized Methyl Linolenate by Sephadex LH-20 and Inhibition of Amidase Activity of β -Trypsin by the Fractions Obtained. Column load : 420 mg of oxd MLn.

● Inhibition, ---- Lipid weight.

inhibition of amidase activity of β -trypsin by the fractions obtained. The oxd MLn was separated into polymers, monomers, and degradation products¹¹. Inhibitory activity of each fraction was assayed as follows. Each fraction, in which eluting solvent had been removed by evaporation under reduced pressure, was again dissolved in 1.0 ml of EtOH. An aliquot (20 μ l) of the EtOH solution was added to 5 μ g of β -trypsin in 180 μ l of 0.1 M Tris-HCl buffer (pH 8.5) containing 0.02 M CaCl₂ and the mixture solution was incubated at 37°C for 90 min. Then, the residual enzyme activity was measured using BAPNA as the amidase substrate¹¹⁻¹³. Among the fractions of degradation products, both inhibitory and potent acceleratory activities were observed around tube Nos. 78~81 and 83~92, respectively. Thus, the degradation products appeared to contain not only amidase accelerators, of which properties had already been reported^{11,12}, but also inhibitors (tube Nos. 78~81 indicated as Fraction I). In order to confirm the generation of these inhibitory substances during the autoxidation of MLn, the active degradation products (ADPs) were further separated from Fraction I by a silicic acid column chromatography¹² (data not shown) and were used for subsequent experiments. The ADPs thus prepared were recovered about 0.2 % from the starting MLn.

3.2 Characterization of Inhibitory Activity of ADPs

Figure 2 shows the effect of conditions for reaction between the ADPs and β -trypsin on

Fig. 2 Effect of Reaction Conditions between the ADPs and β -Trypsin on Inhibition of Amidase Activity of β -Trypsin.

Added amount : β -Trypsin 10 μg , ADPs 100 μg .
Reaction condition : a, pH 8.0, 37 $^{\circ}\text{C}$; b, pH 8.0, 90 min ; c, 37 $^{\circ}\text{C}$, 90 min.

inhibitory activity of the ADPs against amidase activity of β -trypsin. The inhibitory reaction was suggested to be dependent on all of reaction time, temperature, and pH.

β -Trypsin shows not only amidase activity but also esterase and proteolytic activities⁽⁶⁾. We had previously demonstrated that the amidase acceleratory substances, which were observed around tube Nos. 83~92 in Fig. 1, did not significantly influence the esterase and proteolytic activities of β -trypsin⁽¹¹⁾. Therefore, the effect of the ADPs on these activities was examined (Fig. 3). Besides inhibiting the amidase activity, the ADPs could inhibit both esterase and proteolytic activities of β -trypsin, suggesting that β -trypsin is completely inactivated by the ADPs. The inhibitory substances are confirmed to be generated together with the acceleratory ones during the autoxidation of MLn.

Fig. 3 Effect of the ADPs on Esterase and Proteolytic Activities of β -Trypsin.

Added amount : β -Trypsin : 10 μg . After reaction of the ADPs with β -trypsin in 10% EtOH-0.1 M Tris-HCl buffer (pH 8.0)-0.02 M CaCl_2 at 37 $^{\circ}\text{C}$ for 90 min, aliquot of the reaction mixture was used for the assay of each enzymatic activity. ● Esterase activity, ○ Proteolytic activity.

Fig. 4 Effect of Chemical Modification of Functional Groups of the ADPs and β -Trypsin on Inhibition of Amidase Activity.

Added amount : β -Trypsin, Acetylated trypsin 10 μg . Modification of functional groups : Carbonyl group $\rightarrow$ DMH, Hydroxyl group $\rightarrow$ TMS, Hydroperoxide $\rightarrow$ TP-treated ADPs : ϵ -Amino group of trypsin $\rightarrow$ Acetylated trypsin. ○ Control (ADPs + β -trypsin), $\triangle$ DMH + β -trypsin, $\square$ TMS + β -trypsin, ● TP-treated ADPs + β -trypsin, $\blacktriangle$ ADPs + Acetylated trypsin.

Figure 4 shows the effect of chemical modification of the functional groups of the ADPs and trypsin on the inhibition of amidase activity. By converting the carbonyl and hydroxyl groups of the ADPs into DMH and TMS ether derivatives, respectively, the inhibitory activities of the derivatives were markedly reduced. Both functional groups of the ADPs were demonstrated to be

Fig. 5 Inhibitory Activity of the ADPs against Lysozyme and α -Chymotrypsin Activities.

Reaction condition : Lysozyme 1 μ g, pH 8.0, 90 min ; α -Chymotrypsin 25 μ g, pH 8.0, 30 min.

essential for the occurrence of potent inhibition. However, the TP reduction of hydroperoxide(s) to hydroxyl group(s) did not significantly influence the original inhibitory activity of the ADPs. Needless to say, the completion of the TP reduction of hydroperoxide(s) had previously been ascertained by the HPLC analysis described by Nakamura and Maeda¹⁵⁾. Thus, hydroperoxide(s), which has generally been supposed to be injurious to biological systems, was not always involved in the inhibitory activity of the ADPs. On the other hand, ϵ -N-acetylated trypsin, of which all or most of the ϵ -amino groups had been substituted by acetylation¹⁷⁾, was scarcely inhibited by the ADPs, suggesting that the inhibitory action will be associated with amino-carbonyl reaction between carbonyl group(s) of the ADPs and ϵ -amino group(s) of β -trypsin.

Inhibitory activity of the ADPs against other enzymes was examined as the reference samples to assess their injurious activity to biological systems. **Figure 5** shows inhibitory activity of the ADPs against lysozyme and α -chymotrypsin, both of which have often been used for evaluation of inhibitory activity of lipid oxidation products^{4),6),18),19)}. The ADPs strongly inhibited both enzymatic activities as well as β -trypsin, suggesting that the ADPs will behave as inhibitors of various enzymes.

4 Discussion

Lipid oxidation products are well known to damage various kinds of enzymes and thereby inhibiting the activities. On the other hand, there

is an exception that pepsin activity was stimulated by various oxidized products from unsaturated lipids such as linoleic acid⁷⁾, safflower oil⁸⁾, and MLn²⁰⁾. These reports are somewhat different from our studies that oxd MLn could behave as both inhibitor and accelerator of β -trypsin, while pepsin activity was accelerated but not fully inhibited by the autoxidized lipids.

In the previous paper¹¹⁾, the acceleration of amidase activity of β -trypsin was presumed to depend on the amino-carbonyl reaction between the carbonyl groups of the degradation products and ϵ -amino groups of β -trypsin. This reaction is in accordance with that observed in this study, suggesting that the degradation products will display both activities by forming adducts with β -trypsin in the similar mechanisms. However, it is unknown how the ADPs are able to inhibit β -trypsin while some other degradation products promote the amidase activity. In order to understand these interesting phenomena, it may provide the key to elucidate the structural similarities and differences between accelerators and inhibitors occurred during the autoxidation of MLn. The results of the chemical modification of functional groups of the ADPs suggest that the inhibitory substances would be hydroxycarbonyl analogs, which are known to be generated during the autoxidation of polyunsaturated fatty acids^{1),11),14)}. However, our finding inhibitory substances originating from ω 3 polyunsaturated fatty acid MLn must be different from 4-hydroxynonenal, the notorious major toxic hydroxyaldehyde, formed through peroxidation of ω 6 polyunsaturated fatty acids such as arachidonic and linoleic acids¹⁾⁻³⁾. Thus, the exact structures of the inhibitory substances in the ADPs are now under investigations in our laboratories.

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[ノート] リノレン酸メチルの自動酸化中におけるトリプシン阻害 および促進物質の同時発現

—トリプシン阻害物質の確認—

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リノレン酸メチルの自動酸化中に β -トリプシン活性を阻害する一群の分解生成物が、すでに明らかにされているトリプシンのアミダーゼ活性促進物質とともに形成されていることがわかった。阻害作用の発現にはこれら分解生成物のカルボニル基と水酸基ならびにトリプシンの ϵ -アミノ基が不可欠であること、ヒドロペルオキシドは必ずしも関与していないことを官能基の化学修飾実験で示した。このトリプシン阻害活性をもつ分解生成物はリゾチームや α -キモトリプシン活性を強く阻害したことから、各種酵素に対しても阻害物質として作用しうることが推察された。これらのことは不飽和脂質の自動酸化中に各種酵素の阻害物質や促進物質が同時に形成される可能性を示唆している。

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[速報] ポリエチレングリコール鎖を結合させたリン脂質を含む リポソームのフリーズフラクチャー法による電顕写真

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ジステアロイル-N-モノメトキシ-ポリエチレングリコーラスクシニル-フォスファチジルエタノールアミン(PEG-DSPE)を含むジパルミトイルフォスファチジルコリン(DPPC)リポソームのフリーズフラクチャー法による電顕写真を撮影した。DPPC二分子膜の主相転移温度と前転移温度の間の温度から急速凍結させた上記リポソームの断面には帯状構造の他に平面構造も存在していたことからリポソーム内での相分離が示唆された。

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