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Purification and Characterization of Lipoxygenase Inhibitor Produced by *Penicillium* sp.

Ji-Sook Hwang, Yong-Kee Jeong* and Tae-Ho Lee†

Dept. of Microbiology, Pusan National University, Pusan 609-735, Korea
**Dept. of Microbiology, Dongeui University, Pusan 614-010, Korea*

Abstract

A strain of *Penicillium* sp. extracellularly produced an inhibitory substance for lipoxygenase. These purification procedures were followed : ethanol treatment, chromatographies on Dowex 50W, Sephadex G-25, silica gel column and HPLC. The inhibitor was stable in pH range from 3.0 to 5.0 at 25° C, and a treatment at 100° C for 2 hours didn't diminish its original activity. The purified inhibitor was charred at a temperature near 220° C~230° C and decomposed. Molecular weight of the inhibitor was estimated to be approximately 270 by Sephadex G-25 column chromatography. The inhibitor rapidly formed EI complex with lipoxygenase and inhibited enzyme activity.

Key words : lipoxygenase inhibitor, lipoxygenase, *Penicillium*, purification

INTRODUCTION

Lipoxygenase (linoleate : oxygen oxidoreductase, EC 1.13.11.12), known as lipoxidase or carotene oxidase, belongs to a class of dioxygenases which catalyze the peroxidation of fatty acid containing *cis, cis*-1, 4-pentadiene moieties. Lipoxygenases are essentially ubiquitous in eukaryotic organisms and have been found to exist in many tissues of numerous higher plants and animals[1~4], as well as in fungi, yeast, and chlorella[5~7]. The principal substrates for lipoxygenases in higher plants are the di- and tri-unsaturated fatty acids, linoleic (C18 : 2) or linolenic (C18 : 3) acids[8]. Lipoxygenases are thought to have an important role in the formation of flavors and aromas (which may be desirable or not) of many plant products. In some food products the presence of lipoxygenase derives desirable flavors and aromas, but in many food products they are opposite[4,9]. Lipoxygenases are particularly abundant in legume seeds such as soybeans, and oxidize unsaturated fatty acids, resulting in the formation of volatile carbonyl compounds which cause undesirable grassy-beany flavor. Therefore, in food science and plant breeding, a major goal has been to remove or limit soybean seed lipoxygenase activity. Lipoxygenases from soybean seeds have been most thoroughly studied because of their importance to the quality of soybean products. Soybean plant mutants lacking lipoxygenase[10~13], decomposition of the beany flavor by aldehyde dehydrogenase[14~16], and removal of the enzyme substrate with organic solvents[17,18] have been attempted to inhibit the lipoxygenase action and to eliminate the grassy-beany flavor. Recently, antioxidants are widely used as the inhibitors of the autoxidation of unsaturated fatty acid. However, the limited number of safe antioxidants remains when dealing with plant materials intended for human consumption. Therefore, the inhibition of the lipoxygenase by specific inhibitors is expected to be useful in overcoming these problems. The purpose of this study is to isolate microorganisms producing these inhibitors. The results concerning cultivation of an isolated strain as production of its inhibitor were reported in a previous paper[19]. This paper describes the purification and some properties of lipoxygenase inhibitor from a strain *Penicillium* sp. isolated from soil.

† To whom all correspondence should be addressed

MATERIALS AND METHODS

Materials

Lipoxygenase and linoleic acid were purchased from Fluka AG Switzerland. Dowex 50w, Sephadex G-25, blue dextran, FAD, ATP, and ADP were obtained from Sigma(St. Louis, Mo., USA). TLC plate of silica gel was commercial products provided by E. Merck (W. Germany). All other reagents used were of reagent grade.

Cultivation

A loopful of spores from a well sporulated slant culture of *Penicillium* sp. was inoculated in a 500 ml shake flask containing 100ml of seed medium, consisting of glucose 3.0%, polypeptone 1.0%, beef extract 0.1% and potassium phosphate (dibasic) 0.1%. The pH of medium was adjusted to 6.0 before sterilization. The cultivation was carried out for 48 hours at 30° C on a reciprocal shaker (agitation : 120Rev. × 6cm stroke). The seed culture (2.5ml) was transferred into 500ml shaking flasks containing 100ml of production medium, consisting of glucose 3.0%, ammonium sulfate 0.4%, and potassium phosphate(dibasic) 0.1%. The pH was adjusted to 7.0 before autoclaving. The cultivation was carried out for 192 hours at 30° C under the same conditions mentioned above.

Determination of inhibitory activity

Inhibitory activities were expressed in terms of ability to inhibit the activity of lipoxygenase, which catalyzes oxidation of linoleate to linoleic acid hydroperoxide with an absorbance at 234nm[20]. The inhibitor assay was carried out at 25° C in a quartz cuvette with a 1.0cm light path. After preincubating 1.0ml of enzyme solution and 0.1ml of a sample to be assayed for 10min in the cuvette (control contained the same volume of water), 2.0ml of substrate solution was added to the cuvette and inverted 3 times, and the decrease in absorbance at 234nm was recorded for up to 10min. The inhibitory activity was calculated by using the slope of the linear portion of the recorder tracing of a spectrophotometer. The concentration in the assay medium was 0.18M borate buffer, 0.12μl linoleate/ml, and 0.67μg lipoxygenase/ml. A unit of inhibitory activity was defined as the amount of inhibitor which inhibited the fifty percent of enzyme activity, namely IC_{50} value.

Molecular weight determination

According to Andrews[21], molecular weight of the inhibitor was determined by Sephadex G-25 gel filtration with three standard materials, ADP (MW 427.2), ATP (MW 507.2), and FAD (MW 829.5). Two hundred microliters of solution from each of the standard and inhibitor solutions was applied separately on a Sephadex G-25 column (1.5 × 90cm) and eluted with distilled water at a flow rate of 12ml/h, collecting 1ml-fractions. The void volme of the column was determined with Blue-dextran before and after application of the standard substances and sample inhibitor.

RESULTS AND DISCUSSION

Purification of inhibitor

An isolated *Penicillium* species was cultured by shaking reciprocally at 30° C for 8 days. Because the inhibitor was stable at high temperatures, the culture broth was autoclaved to eliminate other impurities including heat labile proteins etc. Heat treated broth was filtered through a filter paper and centrifuged at 12100 × g for 10min. The resulting broth filtrates were concentrated by evaporation and then used as the starting material for the following purification steps.

To the clear filtrate was added absolute ethanol of 2.5 fold volumes to extract the inhibitor and precipitate other impurities which are insoluble in ethanol. After treatment of ethanol, supernatant which is free from ethanol by evaporation was applied to a column of Dowex-50W(28 × 180mm), which was previously equilibrated with 1×10^{-3}M hydrochloric acid solution. After the column was washed with the same solution, the adsorbed inhibitor was eluted with a linear gradient of 0.2M to 2.5M of sodium acetate buffer (pH 5.0). The active fractions from Dowex-50W column chromatography were pooled and concentrated in vacuo. Further purification was

carried out by Sephadex G-25 gel chromatography-(24×940mm) eluted with distilled water and then followed by silica gel column chromatography with a solvent system consisting of *n*-butanol, acetic acid and water (5 : 1 : 4, v/v). Final purification was done with semipreparative HPLC on C_{18} reverse phase column (Waters, μbondapak-phenyl) eluted with 10% methanol. The steps leading to the isolation and purification of the inhibitor from the culture broth were summarized in Fig. 1. The inhibitor solution eluted by HPLC displayed one peak on a chromatogram of HPLC (Fig. 2), and also proved to be homogeneous by thin layer chromatography using various developing solvent systems (Fig. 3). The powder lyophilized by a freezing dryer was brownish and rapidly damped when exposed to the air. The inhibitor is soluble in water, ethanol, and methanol, but never in nonpolar organic solvents such as benzene

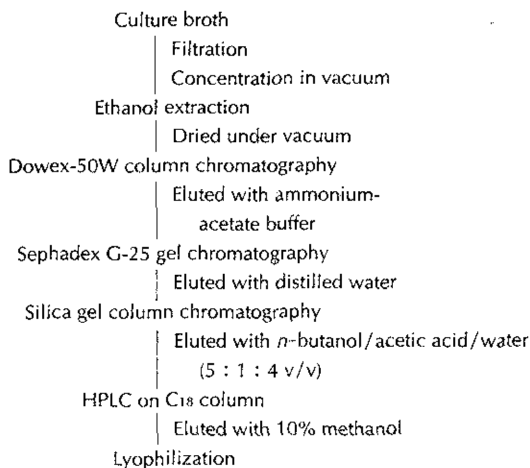

Fig. 1. Purification scheme of the lipoxygenase inhbitor.

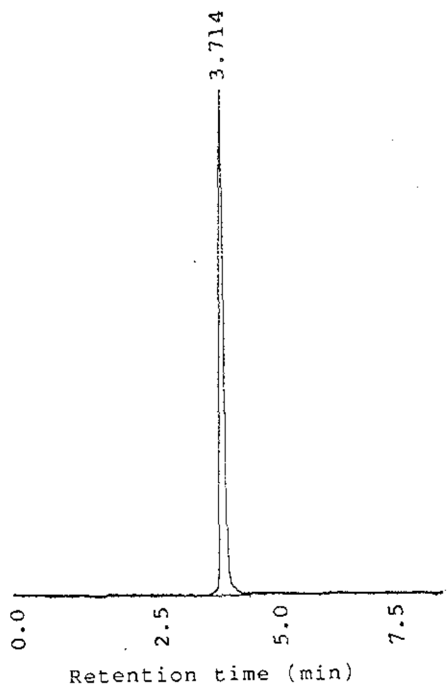

Fig. 2. High performance liquid chromatography of lipoxygenase inhibitor.
Column : Waters μbondapak TM phenyl
Eluting solvent : MeOH : H_2O (1 : 9)
Flow rate : 1.0ml/min
Detector : UV detector model 441, 313nm

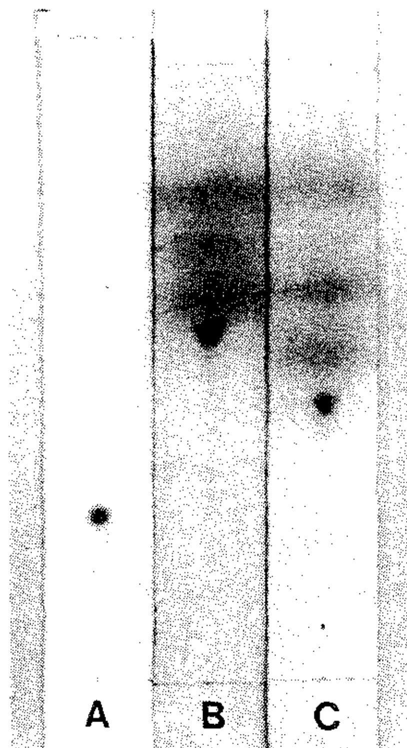

Fig. 3. Thin layer chromatography on silica gel G-60.
The spot was identified by sulfuric acid.
Developing solvent systems were as follows.
A, *n*-butanol : acetic acid : water (5 : 1 : 4).
B, ethanol : ammonium hydroxide : water (6 : 3 : 1).
C, isopropanol : ammonium hydroxide : water (6 : 3 : 1).

and chloroform.

Properties

The absorption pattern of the purified inhibitor is shown in Fig. 4. The inhibitor showed maximum absorption near 310nm, but didn't absorb at a visible wavelength.

In order to determine the molecular weight of the inhibitor, we ran gel filtration of the purified inhibitor on Sephadex G-25. As shown in Fig. 5, the molecular weight of the inhibitor was estimated to be about 270.

The purified inhibitor was treated at different pHs from 1.0 to 11.0 for 24 hours at 25°C, and then the remaining activity was measured. In Fig. 6, the inhibitor was stable in acidic pH range, but above pH 5.0 it rapidly lost its activity. To investigate temperature stability of the purified inhibitor, the inhibitor was dissolved in 0.2M citrate buffer (pH 3.0) and treated at various temperatures for 30min, and then the residual activity was measured. As a result, the activity of the inhibitor remained intact after incubation at 100°C. Even after the inhibitor was heated for 30min on the autoclave, more than 90% of the activity remained ; so the purified inhibitor proved very stable at temperature.

The correlation between the concentration of the inhibitor and its inhibitory activity against lipoxygenase is shown in Fig. 7. The IC_{50} value of the purified inhibitor against soybean lipoxygenase was about 32μg(0.118μM), lower than those of turmeronol A and turmeronol B from the spice tumeric and soybean lipoxygenase inhibitors, in which IC_{50} occurs at 17μM and 9μM, respectively[22].

We investigated the melting point of the purified inhibitor. However, the purified inhibitor did not melt, but decomposed near 220°C~230°C.

To seek the characteristics of the purified inhibitor, we investigated its various biochemical reactions. The results (not shown) were positive to Ninh-

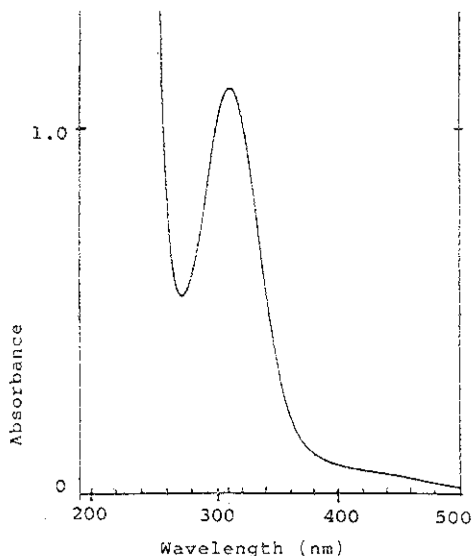

Fig. 4. The ultraviolet absorption spectrum of purified lipoxygenase inhibitor.

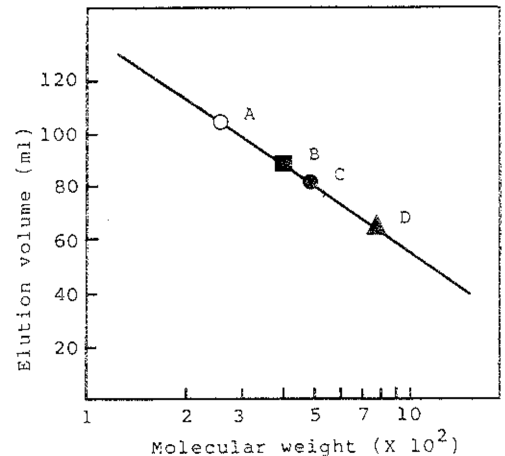

Fig. 5. Determination of the molecular weight of inhibitor by Sephadex G-25 gel.
A : lipoxygenase inhibitor, B : ADP, C : ATP, D : FAD

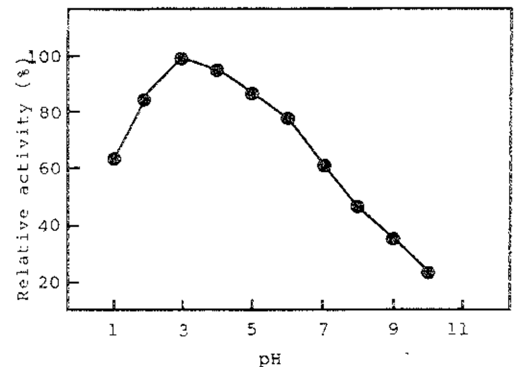

Fig. 6. Effect of pH on stability of lipoxygenase inhibitor.

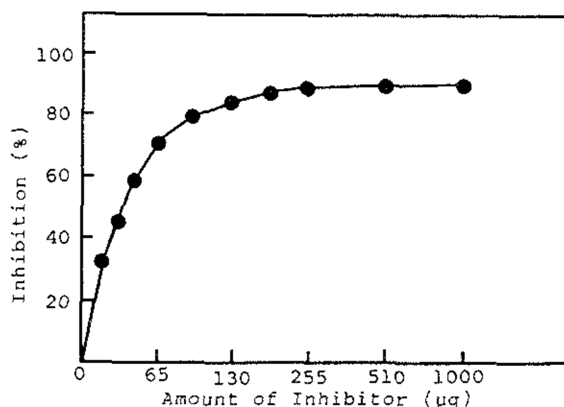

Fig. 7. The effect of the concentration of the inhibitor on the soybean lipoxygenase.
After 1ml of lipoxygenase solution was preincubated with 0.1ml of inhibitor solution at 30°C for 10min, 2ml of linoleic acid solution was added and incubated at 30°C, 10min, and then residual lipoxygenase activity was assayed.

ydrin, Xanthoprotein, Somogyi-Nelson test, Fehling test, Tryptophan-sulfuric acid and 2,4-Dinitrophenyl hydrazine, but negative to Biuret test, Barfoed test and Bromocresolgreen reagent. Judging from these results, we suppose that the purified inhibitor may be a heat stable substance having the aldehyde and amino groups, and now under analysis of composition and structure of the inhibitor.

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Penicillum sp. 에의해 생산되는 Lipoxygenase Inhibitor의 정제 및 성질

황지숙 · 정영기* · 이태호[†]
부산대학교 미생물학과
*동의대학교 미생물학과

요 약

토양으로 부터 분리한 lipoxygenase inhibitor 생산균주 *Penicillium* sp.를 배양하여, 배양액으로 부터 저해물질을 분리, 정제하였다. 배양액을 여과한 후 원심상등액을 농축하고, ethanol 침전에의해 불순물을 제거하였다. Ethanol은 감압농축기로 제거한 후 Dowex 50W, Sephadex G-25, silica gel column 및 HPLC 등으로 single peak를 나타낼때까지 정제하였다. 정제된 본 저해제는 산성 영역에서 비교적 안정하였으며, 열 안정성이 특히 우수하여 100°C에서 2시간의 가열에 활성이 그대로 유지되었다. 분자량은 약 270으로 측정되었으며, 220°C~230°C 부근에서 용융되지 않고 decomposition 되는 성질을 나타내었다. 저해제는 lipoxygenase와 complex를 형성하여 효소를 효율적으로 저해하였다.