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Isolation and Cultivation of Microorganism Producing Lipoxygenase Inhibitor

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Lipoxygenase Inhibitor를 생산하는 미생물의 분리 및 배양

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ABSTRACT

The microorganisms producing a lipoxygenase inhibitor were screened from a wide variety of sources. The isolated strain was assigned to genus *Penicillium* by its cultural and morphological characteristics. The proper medium for the production of lipoxygenase inhibitor was composed of glucose 3.0%, ammonium sulfate 0.4%, and potassium phosphate (dibasic) 0.1%. The cultivation for lipoxygenase inhibitor production was carried out in 500ml Erlenmyer flasks containing 100ml of the medium at 30℃ by cultivating reciprocally. The highest lipoxygenase inhibitor production was observed after 8 days of cultivation. The inhibitor was the low molecular weight substance and inhibited specifically soybean origin lipoxygenase.

INTRODUCTION

Soybeans are potentially the most abundant and economical source of high quality vegetable protein. The wide use of many soybean protein preparations, particularly soymilk, is limited due to undesirable flavor and odor. These undesirable flavor and odor are principally caused by an enzyme, lipoxygenase. Lipoxygenase (linoleate ; oxygen oxidoreductase, EC 1.13.11.12.) catalyzes the hydroperoxidation of unsaturated fatty acids and esters containing a cis-cis-1, 4-pentadiene system by oxygen molecule.

Soybean seed contains a large quantity of lipoxygenase which oxidizes the useful unsaturated fatty acids during food processing, resulting in the formation of volatile carbonyl compounds(1) which cause undesirable green, beany, and grassy odors(2, 3, 4) and sour, bitter, and astringent tastes(5, 6). Therefore, inactivation of soybean lipoxygenase can eliminate the formation of these undesirable flavors in the soybean protein preparations. Wilkens *et al.*(7) eliminated off-flavor development in soymilk by grinding the unsoaked dehulled beans in water heated from 80 to 100℃ for 10 min. Mustakas *et al.*(8) obtained thei simi-

lar results by subjecting dehulled soybeans to heat at 100℃ or to steam for drying. Although the heat treatment inactivated effectively lipoxygenase, other proteins are also denatured and losed solubility and functionality in foods. Loss of functionality is another deterrent to use soy-products. Borhan *et al.*(9) investigated by the combined treatment of heat and ethanol on soybeans to see whether lipoxygenase activity was destroyed and the considerable protein solubility and functionality were still maintained or not. This method was proper to destroy lipoxygenase and maintain protein functionality; however, the concomitant loss of protein solubility and the impartation of a cooked soybean flavor were still remained(8, 10, 11). The development of a general lipoxygenase inhibitor seems to be an ambitious plan for overcoming these problems. The antioxidants and substrate analogues, such as milk casein(12), sulfhydrazine(13), Turmeronol(14), and polyunsaturated fatty acid (15), have been known as lipoxygenase inhibitor for these purposes. However, these inhibitors are chemically synthesized, and unstable compounds when exposed to air. In addition, some inhibitors have never been practically used for such reasons as their toxicity, cost, and weak activity. Therefore, we attempted to isolate a microorganism producing a specific inhibitor of soybean lipoxygenase and succeeded in isolating a fungi from soil. The isolated strain was assigned to genus *Penicillium* from its cultural and morphological characteristics. This paper deals with the characteristics of a microorganism isolated from soil and conditions for the production of lipoxygenase inhibitor.

MATERIALS AND METHODS

Isolation of microorganisms producing lipoxygenase inhibitor

Soil samples were collected from several sites in Korea. These soil samples were dispersed in sterile water and one loop of a soil suspension was spread on medium. The plates were incubated at 30℃ for 3 days and the developed colonies was transferred to a slant medium. The isolates were cultivated for 3 days with shaking at 30℃, and then each broth was heated for 10 min at 100℃ and dialyzed to isolate the microorganism which produces a heat stable and low molecular weight inhibitor. After these treatment, the lipoxygenase inhibitory activities from the outside fraction of dialysis bag were measured.

Estimation of the inhibitory sctivity on lipoxygenase

In this study, inhibitory activities against lipoxygenase were conveniently assayed in terms of its ability of catalyzing the oxidation of linoleate to linoleic acid hydroperoxide, which has a maximum absorbance at 234 nm (16). The standard inhibitor assay was carried out at 25℃ in a quartz cuvette with a time-scanning recording. After preincubation of enzyme solution and inhibitor for 10 min in cuvette, the substrate solution was added to the cuvette, inverted 3 times, and the decrease in absorbance at 234 nm was recorded for up to 10 min. The reaction mixture contained 0.18M borate buffer(pH 9.0), ethanol(67 μl), linoleic acid(0.11 μl), lipoxygenase(0.67 μg), and inhibitor solution in a final volume of 3.0 ml. The inhibitory activity was calculated by using the slope of the linear portion of the recorder tracing of spectrophotometer. A unit of inhibitor was defined as the amount of inhibitor which inhibit fifty percent of enzyme activity, namely IC_{50} value.

Identification of strain L242H producing lipoxygenase inhibitor

The purely isolated strain L242H was examined culturally and microscopically. During incubation at 30℃ on Czapek-Dox agar for 15 days, we observed the cultural characteristics including cell growth, changes of colony color, the shape of colony surface, the production of pigment and so on. Morphological characteristics were observed microscopically including the presence of the septum, the shape of conidiophore and sterigmata,

the shape, color and arrangement type of conidia during slide culture on Czapek-Dox agar for 7 days. Taxonomic study on isolated strain was carried out according to the methods of Raper and Thom (17) and Ainsworth (18).

Measurement of cell growth

The cell growth was determined by weighing the dry weight of cells by the following procedure. Culture broth was filtered by filter paper (Toyo filter paper No. 2) followed by washing 3 times with distilled water,and then cells were dried at 105℃. The dry weight of cells was measured when it comes to be an uniform weight.

RESULTS AND DISCUSSION

Selection of microorganism producing lipoxygenase inhibitor

More than 480 strains of microorganisms including fungi, bacteria, and actinomycetes were isolated as candidates for the production of lipoxygenase inhibitor from soil samples and each strain was tested for its ability to produce the inhibitor. About 6 strains of actinomycetes and 7 strains of fungi showed the activities of lipoxygenase inhibitor. Among these, a fungal strain, designated L242H, showed the highest productivity of lipoxygenase inhibitor. The inhibitor produced by L242H strain was heat stable and had the low molecular weight. Therefore, this L242H strain was selected as the representative strain.

Taxonomical studies of strain L242H

The cultural and morphological characteristics of strain L242H are shown in Table 1. The isolated strain L242H grew well on Czapek-Dox agar medium, showed a uniformly velvety colony texture, and produced yellowish brown pigment in the colony reverse.

The color of colony was white and changed into green in accordance with the time of culture. In microscopic morphology of strain L242H (Fig. 1), it produced aerial mycelium and septum. Conidiophore was bivertically asymmetric. Sterigmata was clavate and conidia was ellipsoid. From these results, we decided that the strain L242H belongs to the genus *Penicillium*. Consequently, strain L242H was designated as the name of *Penicillium* sp. L242H.

Table 1. Cultural and morphological characteristics of strain L242H.

Factor	Characteristics
Rate of growth	Growing rather rapidly(spreading)
Colony color	White
Conidia color	Dark green
Character of growth	Velvety
Colony reverse	Yellowish brown
Hyphae	Aerial
Septum	Forming
Conidiophore	Biverticillate, asymmetry
Sterigma	Clavate
Conidia	Ellipsoid
Conidiogenesis	Phialo

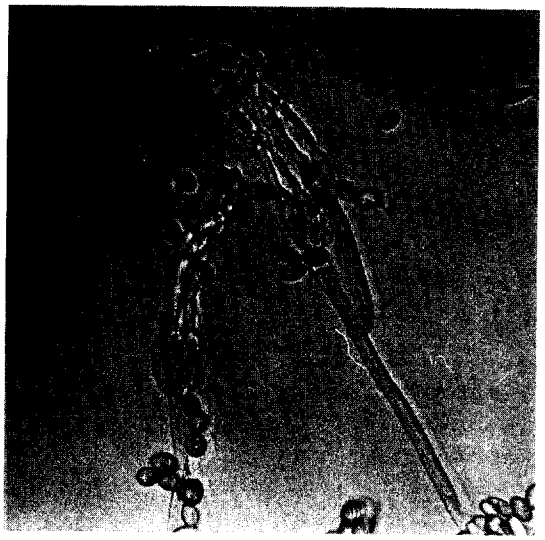

Fig. 1. Microscopic photograph of strain L242H on Czapek-Dox agar after 5 days of slide culture at 30℃.

Cultural conditions for the production of inhibitor

The effect of various carbon sources on the

Table 2. Effect of carbon sources on the production of lipoxygenase inhibitor.

Carbon sources	Final pH	Growth (mg/ml)	Lipoxygenase inhibitor activity(units/ml)
None	6.2	2.05	15.8
L-Arabinose	5.1	12.49	84.2
D-Fructose	3.7	13.86	60.5
D-Galactose	3.4	0.90	39.5
Glucose	5.0	13.11	73.7
Dq-Mannose	3.6	14.60	75.3
Xylose	5.2	11.27	67.5
Glycerine	2.3	16.89	55.3
Inositol	4.0	12.83	16.5
Mannitol	3.0	11.80	28.2
D-Sorbitol	3.2	11.55	35.0
Lactose	8.7	0.06	4.7
Maltose	4.9	14.64	54.1
Sucrose	3.4	15.57	32.5
Dextran	4.9	4.57	34.2
Soluble starch	5.3	12.87	25.0

Table 3. Effect of nitrogen sources on the production of lipoxygenase inhibitor.

Nitrogen sources	Final pH	Growth (mg/ml)	Lipoxygenase inhibitor activity(units/ml)
None	2.3	0.08	0.0
Ammonium acetate	1.8	3.95	49.4
Ammonium chloride	1.8	6.70	18.8
Ammonium nitrate	2.2	7.51	35.3
Ammonium phosphate (dibasic)	1.8	10.02	30.6
Ammonium sulfate	1.8	2.95	75.3
Nitric acid	0.7	0.12	0.0
Potassium nitrate	2.2	4.64	21.4
Sodium nitrate	2.3	3.85	33.3
Sodium nitrite	5.3	0.44	4.8
Urea	2.8	5.86	38.1
Polypeptone	1.7	3.66	31.0
Yeast extract	1.6	3.30	35.7
Beef extract	1.6	3.25	18.4
Polypeptone 1.0% + Beef extract 0.1%	5.2	8.89	73.8

production of lipoxygenase inhibitor was examined(Table 2). The cells were cultivated for 8 days in the media containing each carbon source and the inhibitor activity in the broth was measured. When monosaccharides were added to the medium as a carbon source, relatively large amount of inhibitor were produced regardless to the number of carbon. Except for lactose, disaccharides were also effective on the production of the inhibitor. On the contrary, oligosaccharides showed low productivity of the inhibitor. L-arabinose, glucose, and mannose were the most effective on the production of inhibitor among examined carbon sources. In economical aspects, we determined glucose as a carbon source on the production of lipoxygenase inhibitor and its optimal concentration was 3.0% (w/v).

The effect of various nitrogen compounds was examined in the media with the addition of various nitrogen sources(Table 3). Especially ammonium sulfate or the combination of 1.0% polypeptone and 0.1% beef extract exhibited the good productivity of the inhibitor. Therefore, the combination of 1.0% polypeptone and 0.1% beef extract was supplied as a nitrogen source of seed culture and ammonium sulfate was determined as a nitrogen source for the production of lipoxygenase inhibitor. L242H strain showed the maximum inhibitor activity at a concentration of 0.4% (w/v) ammonium sulfate.

In order to investigate the effect of inorganic salts on the production of lipoxygenase inhibitor, many kinds of inorganic salts were tested by addition to the medium containing 3.0% glucose, 0.4% ammonium sulfate(Table 4). Among the tested several inorganic salts, 0.1% potassium phosphate(dibasic) was selected as an inorganic salt for inhibitor production.

The effect of initial pH of the medium on the production of inhibitor was tested(Table 5). The maximum production of inhibitor was obtained when initial pH of the medium was 7.0; however, the strain L242H grew well over a wide range of pH 4.0~8.0.

Since temperature influenced on the growth of microbes, the the temperature on the production of inhibitor was investigated. As a result, the cultivation at 30℃ was proper to inhibitor produ-

Table 4. Effect of inorganic salts on the production of lipoxygenase inhibitor.

Inorganic salts	Final pH	Growth (mg/ml)	Lipoxygenase inhibitor activity(units/ml)
None	2.0	1.69	0.0
$ZnCl_2$	3.2	0.14	23.8
$HgSO_2$	4.2	0.25	26.2
$MgCl_2 \cdot 6H_2O$	1.9	2.53	28.6
$MnCl_2 \cdot 4H_2O$	1.9	2.22	9.5
$CaCl_2$	1.8	1.47	42.9
$FeCl_3 \cdot 6H_2O$	2.2	1.56	48.5
NaCl	2.0	1.92	42.4
KCl	1.9	1.56	30.3
K_2HPO_4	1.8	2.59	56.6
KH_2HPO_4	1.8	2.17	26.3

Table 5. Effect of initial pH of medium on the production of lipoxygenase inhibitor.

Initial pH	Final pH	Growth (mg/ml)	Lipoxygenase inhibitor activity(units/ml)
4.0	1.7	3.82	44.4
5.0	1.7	2.94	57.8
6.0	1.7	3.32	57.8
7.0	1.7	2.48	80.0
8.0	1.7	4.16	62.2

Table 6. Effect of cultivation temperature on the production of lipoxygenas inhibitor.

Cultivation temperature(℃)	Final pH	Growth (mg/ml)	Lipoxygenase inhibitor activity(units/ml)
20	1.8	2.27	80.0
25	1.7	2.37	96.5
30	1.7	3.17	106.0
35	1.9	3.58	40.0
40	2.3	1.44	19.6

ction(Table 6). Through the investigation of the relation between the aeration and the productivity of inhibitor (data not shown), we found out that the inhibitor productivity was proportion to the aeration to some extent, whereas cell growth was in inverse proportion to the aeration. The high production of inhibitor was achieved at 100ml of the medium in 500ml flask.

Table 7. The optimum culture condition for the production of lipoxygenase inhibitor.

Medium	Glucose	3.0%
	Ammonium sulfate	0.4%
	Potassium phosphate (dibasic)	0.1%
	Initial pH	7.0
Other conditions	Temperature	30℃
	Culture time	8 days
	Agitation	120 Rev. X 6cm stroke (reciprocal)

100ml of medium per 500ml shaking flask

The establised optimal cultural conditions for the production of inhibitor are summarized in Table 7.

Time course of the inhibitor production

The inhibitory activity, pH, and cell growth during cultivation with the optimized medium were monitored at regular intervals. The seed culture grown in a 500ml Erlenmeyer flask for 2 days on a reciprocal shaker was inoculated into 100ml of the optimized medium at a concentration of 2.5% (v/v). As shown in Fig. 2, the inhibitor biosynthesis occured at the exponential phase. The inhibitor was apparently produced during active growth and there was a close linear relationship between inhibitor formation and the increase of growth. The inhibitor accumulation in the culture broth reached maximum after 8 day cultivation and rapidly decreased during the stationary phase. For isolation of the compound, cultivation was terminated at 8 days.

Inhibitory activity of crude inhibitor

Inhibitory activity of crude inhibitor obtained from optimal cultural conditions was investigated according to its arbitrary concentration. As appeared on Fig. 3, crude inhibitor effectively inhibited the activity of enzyme to some extent, but not above 80%. Therefore, we decided that crude inhibitor must be purified and be qualitatively tested its inhibitory activity according to its concentration, and are now on purification.

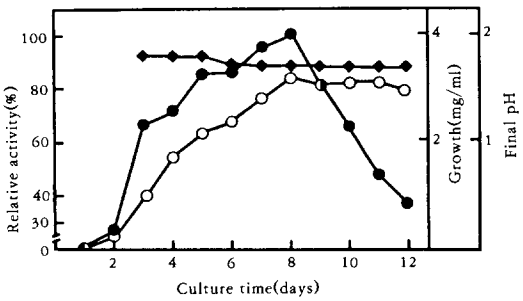

Fig. 2. The time course of cultivation of strain L242H. *Penicillium* sp. L242H was cultivated in optimum culture condition described in Table 7. The pH, inhibitor activity, cell growth during cultivation were measured. Inhibitor activity; ●—●, Cell growth; ○—○, Final pH; ◆—◆

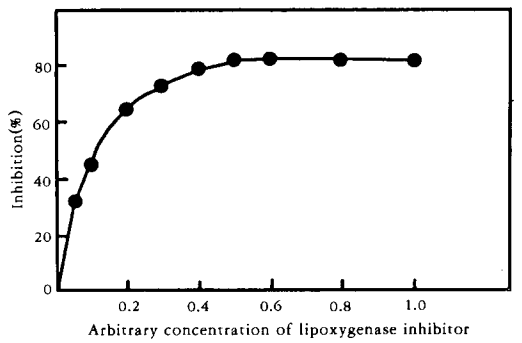

Fig. 3. Inhibitory effect of crude inhibitor solution on the lipoxygenase. One ml of lipoxygenase solution was preincubated with 0.1ml of crude inhibitor solution at 30℃ for 10mins, and then 2ml of substrate solution was added and incubated at 30℃ for 10 mins. The residual enzyme activity was assayed.

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요 약

토양으로부터 세포외로 lipoxygenase inhibitor를 생산하는 균주를 screening하여 우수한 저해능을 가지는 곰팡이 1균주 (L242H)를 분리하였고, 분리한 균주 L242H로부터 lipogenase inhibitor 생산조건을 검토하였다. 분리한 균주의 배양학적 · 형태학적 특성을 조사해 본 결과 *Penicillium*속으로 동정되었다. *Penicillium* L242H의 플라스크 배양에 의한 lipoxygenase inhibitor 최적배양조건은 3.0% glucose, 0.4% ammonium sulfate, 0.1% potassium phosphate (dibasic), 초기 pH 7.0이었고, 500ml 용 shake flask에 배지 100ml를 넣어 8일간 배양했을 때 lipoxygenase inhibitor가 최대로 생산되었다.

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