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Lipase Inhibitors from Green Pepper, *Capsicum annuun* Lin.

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피만 (*Capsicum annuun* Lin.)중의 Lipase Inhibitors

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Introduction

Brockerhoff(1969) established that pancreatic lipase had the unusual property of being functional only at an oil/water interface and that the hydrolysis reaction was dependent upon the interfacial area rather than the bulk concentration of the substrate.

Any substances which can alter the nature of the oil/water interfaces can also markedly influence the hydrolysis of triglycerides.

Among the substances, the following materials have been long reported as inhibitors; (1) chemical reagents (Bier, 1955) such as a variety of ketone and aldehyde, heavy metals, halogen ions, alkaloids, alcohols, fluorophosphate, chloroform, bromoform, formaldehyde, lactones, etc., (2) SH-blocking reagents (Sanger, 1948) such as *p*-chloromercuric benzoate, trivalent organic arsenicals, *o*-iodosobenzoate and ferricyanide, (3) the mixed substrates (Bier, 1955) showing a competitive inhibition, (4) soaps of long chain fatty acids (Sugiura and Ogiso, 1970) showing the competitive inhibition in very low concentration and the reduction of inhibition by bile salts or Ca^{2+} ion.

On the other hand, it has been recently attached importance to the natural enzyme inhibitors which are the macromolecular substances found in a variety of plant tissues.

The interest in these inhibitors stems from not only the academic importance but also the economic importance of the possible utilization of inhibitors in medical, nutritional, and food technological aspects rather than chemical sustances that are currectly used (Pressey, 1972).

As the natural lipase inhibitors, Brandle (1970) separated protease from microbes or vegetables, Hochstrasser *et al.* (1972) lipid from seeds of *Arachis hypogaea*, Milic *et al.* (1972) tannins from lucerne, and Satouchi *et al.* (1974) inhibitor protein from soybean seeds.

The author found that green peppers contained some materials inhibiting pancreatic lipase in a preliminary experiments.

This study was undertaken to separate and purify the lipase inhibitors from green peppers and to elucidate the properties and inhibition mode of purified inhibitors.

Separation and Some Properties of Crude Inhibitor

In the separation procedure, the following facts were found: (1) the lipase inhibitors were soluble in water as well as in the various concentrations of salt solution, (2) the lipase inhibitors were precipitated

Table 1. Fractionation of crude extracts with ammonium sulfate

Fractions	Total OD (at 280 nm)	Spec. Act. ($1/OD_{23}$ at I_{50})	Total Act. (per kg of sample)
0.2	23.6	0.244	5.7
0.4	82.6	0.742	61.3
0.6	204.4	0.813	166.2
0.8	1524.6	0.829	1263.9
1.0	1489.6	0.385	573.5
Super	1549.2	0.013	20.1

with high concentration of ammonium sulfate, (3) the inhibitory activity tended to decrease by the stirring which seemed to cause the oxidative degradation or denaturation during extraction.

Thus, attempts were made successfully to separate the crude lipase inhibitors from green peppers by the extraction without stirring with 0.85 M NaCl solution which was the most effective among the solvents examined and by the fractionation of the extracts with 0.6-1.0 saturation of ammonium sulfate (Table 1).

The crude lipase inhibitor preparation separated has the following properties: (1) the crude preparation was stable to 30 min incubation at the temperatures below 50°C and at a pH range from 3 to 10, (2) the crude preparation did not require metal ions to exhibit its inhibitory activity, (3) the crude preparation inhibited not only the activity of pancreatic lipase but also that of rice bran lipase, and (4) the crude preparation inhibited the activity of pancreatic lipase at high concentrations but rather accelerated at a very low concentration (Fig. 1).

These evidences may indicate that the crude lipase inhibitor is not a simple protein but a complex containing certain stable components beside protein, and lacks species-specificity of lipase inhibition.

However, there are some questions as to the specificity of the inhibitor: (1) How contribute the components to the formation of active site of inhibitor, (2) Whether the lack of specificity and the variability of inhibition depending upon inhibitor concentrations are resulted from only the specific characters of the inhibitor or from the effects of various components contained in the crude inhibitor.

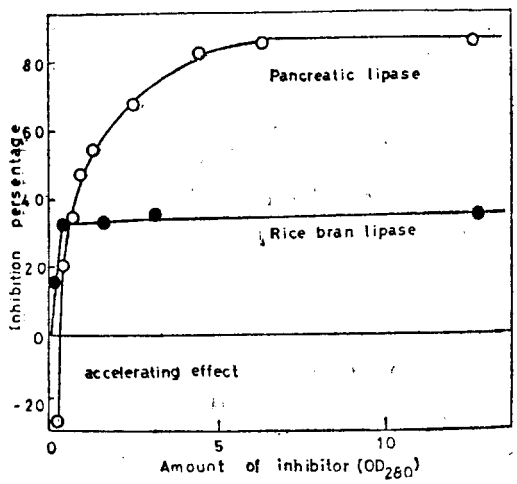

Fig. 1. Relation between inhibitory activity and amount of inhibitor. To each enzyme solution containing 1.0 ml of 0.5 M KCl, 1.0 ml of 5×10^{-3}M $CaCl_2$ and 1.5 units of pancreatic lipase or 0.5 units of rice bran lipase, the various amounts of inhibitor were added and made up the total volume to 9.8 ml with deionized water. After an incubation for 10 min at 35°C, each remaining lipase activity was estimated using 0.2 ml of tributyrin as a substrate.

Purification of Lipase Inhibitors

The examination of purification procedure of the lipase inhibitor revealed the following chemical properties: (1)the inhibitor was precipitated with a high concentration of cold acetone without serious damage of the activity, whereas the inhibitory activity disappeared by the treatment of methanol-ether (1 : 1) mixture, (2) the inhibitor was not adsorbed by ion exchangers such as DEAE-cellulose, CM-cellulose, and SE-cellulose. Moreover, the inhibitor losts considerably its activity, when it was passed through these ion exchangers other than DEAE-cellulose. In the case of DEAE-cellulose, the specific activity of inhibitor increased and the recovery of activity was also improved, (3) when the crude lipase inhibitor preparation was passed through adsorbing agents such as active carbon and hydroxyapatite to remove the slight yellow color components contained in inhibitors, the inhibitory activity was also abolished, (4) the crude proved preparation was more effectively divided into

Table 2. Summary of separation and purification procedures

Fractions	Total OD (at 280 nm)	Spec. Act.	Recovery (%)
Crude extract	125427.0	0.312	100.0
Crude inhibitor	20570.0	1.123	59.0
A70-90	7382.2	1.404	26.5
AD	1665.5	3.650	15.5
ADG	911.4	5.011	11.7
ADRGI	125.1	7.578	2.4
Inhibitor I	65.8	6.745	1.1
ADRGII	708.7	5.741	10.4
L. inhibitor II	497.7	4.875	6.2

two fractions, ADRGI and ADRGII, by twice gel-filtration through Sephadex G-100 than through Sephadex G-25, 50, 75, and 200, (5) ADRGII-fraction was stable on heating, hence it was purified by removing the unstable contaminations in heat treatment, while ADRGI-fraction was unstable in heating, hence it was purified by the repeated gel-filtration through Sephadex G-100.

Thus, the purification of lipase inhibitors were carried out according to the following procedures: (1) the fractionation of crude inhibitor preparation with 70-80% of cold acetone, (2) DEAE-cellulose column-chromatography with 0.01M phosphate buffer (pH 7.0), (3) gel-filtration through Sephadex G-100 with 0.01M phosphate buffer (pH 7.0), (4) regel-filtration through Sephadex G-100 with 0.01M phosphate buffer (pH 7.0), (5) the repeated gel-filtration of ADRGI-fraction through Sephadex G-100 with 0.01 M phosphate buffer(pH7.0) and heat treatment of ADRGII-fraction at 100°C for 10min.

However, the specific activities of purified lipase inhibitors did not so greatly increase compared with that of crude inhibitor (Table 2). This might be caused by removal or denaturation of active components of inhibitors during the purification.

Homogeneity and Physico-Chemical Properties of Purified Lipase Inhibitor I and II

The purified ADRGI-and ADRGII-fractions, referred to as Lipase inhibitor I and II, respectively, were demonstrated to be homogeneous by 7.5% polyacrylamide gel electrophoresis (pH 4.3) with or without sodium dodecyl sulfate (SDS) and by sedimentation experiment (Fig. 2, 3, and 4).

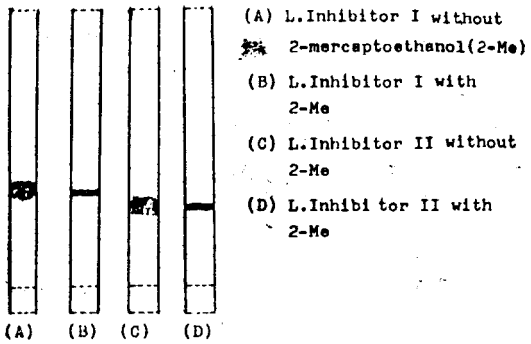

Fig. 2. Polyacrylamide gel disc electrophoretic patterns of purified lipase inhibitors. Electrophoresis was carried out by applying a current of 2~5 mA per tube on 7.5% polyacrylamide gel (pH 4.3)

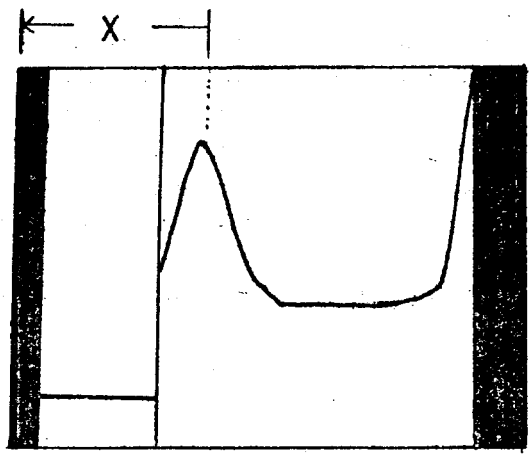

Fig. 3. SDS-polyacrylamide gel electrophoretic patterns of purified lipase inhibitors. Lipase inhibitor (about 50 μg as protein) was denatured at pH 7.2 in 1% SDS solution with or without 1% 2-mercaptoethanol. Electrophoresis was performed at the current of 8 mA per tube on 10% polyacrylamide gel containing 0.1M phosphate butter (pH 7.2) and 0.1% SDS using 0.01% bromophenol blue (BPB) as an indicator of leading ion.

The physico-chemical properties of purified lipase inhibotors are summarized as follows: (1) the sedimentation coefficient of purified lipase inhibitor II was evaluated to be 2.34 S, (2) the molecular weights of the purified lipase inhibitor II were evaluated as

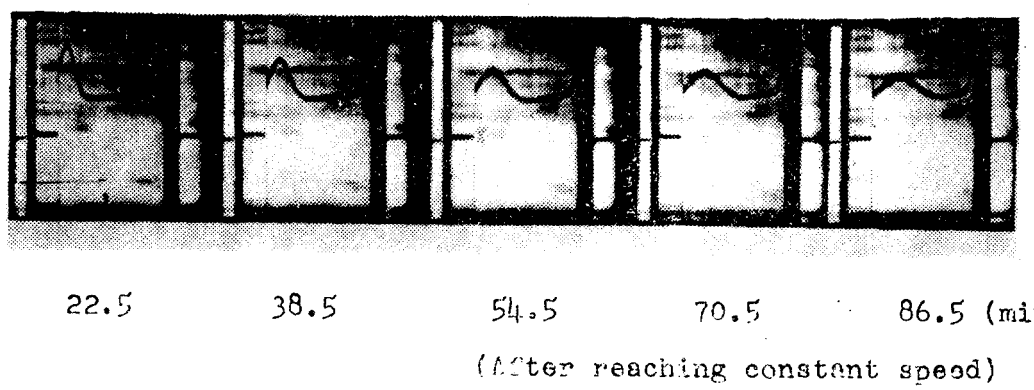

Fig. 4. Sedimentation patterns at 59,780 rpm. Ultracentrifugation was performed by the use of 12mm conventional cell in the Model E Ultracentrifuge manufactured by Spinco Division, Beckman Instruments, Inc., Belmont, California.

23,000 and 30,000 according to SDS-polyacrylamide gel electrophoresis and the method of Archibald (1947), respectively, while the molecular weights of purified lipase inhibitor I were evaluated as 26,000 according to SDS-polyacrylamide gel electrophoresis, (3) the ultraviolet absorption spectra of purified lipase inhibitor I and II were different from that given by a simple protein. Partifularly, in those spectra, a rather greater absorption was observed at the wave lengths below 280nm compared with the optical absorption at 280nm(Fig. 5), (4) in the ampholine electrophoresis (Fig. 6), the protein component of purified lipase inhibitor II focused in the pH range from 8.5 to 10.0, indicating the isoelectric point of near 9.2. However, the focused protein component did not have any inhibitory activity.

From the evidences mentioned above, the following conclusions were drawn: (1) the protein component of purified lipase inhibitor II is basic, (2) the pufified lipase inhibitor II contains certain components which have high optical absorption at below 280nm and the certain components might be acidic counterpats confronting with the basic protein of inhibitor, and (3) the difference between the molecular weight of purified lipase inhibitor II estimated according to SDS-polyacrylamide gel electrophoresis and that according to the method of Archibald might be due to the inaccurate move through SDS-polyacrylamide gel column influenced by the components besides protein.

However, the question has been raised whether the inhibitory activity of purified lipase inhibitor II de-

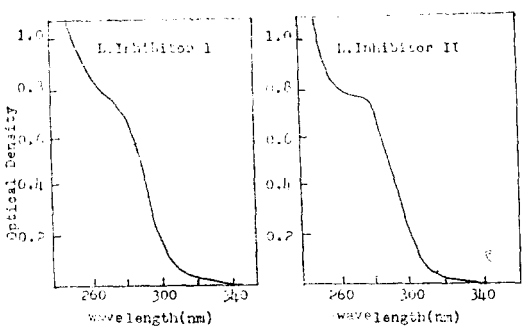

Fig. 5. Ultraviolet absorption spectra of the lipase inhibitors. Cary Model 14 Recording Spectrophotometer was used

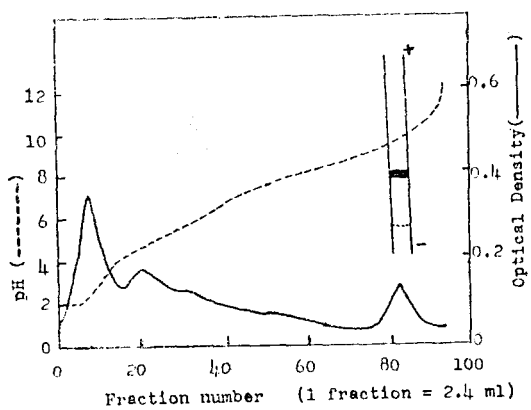

Fig. 6. Isoelectrofocusing pattern of purified lipase inhibitor II. Isoelectrofocusing was conducted at 1.1 watt and 4°C for 50 hours in the presence of 0.8% carrier ampholyte (pH 3.5—10) medium.

pended upon the acidic counterparts alone or the complex of basic protein with the acidic counterparts.

Chemical Properties of Purified Lipase Inhibitor I and II

The chemical compositions of purified lipase inhibitors can be summarized as follows: (1) the inhibitor I was composed of the almost equal amounts of protein and sugar, while the inhibitor II was composed of sugar as the major component and protein as the minor component (Table 3), (2) the sugar component of inhibitor II was mainly consisted of glucose together with a small amount of ribose and sugar not identified (Fig. 7). The unidentified sugar might be sugar acid which was corresponded to the acidic counter parts of inhibitor together with RNA, (3) the protein component of inhibitor II was consisted of ca 14% of basic amino acids, ca 20% of acidic amino acids, ca 32% of non-polar amino acids and ca 10% of S-containing amino acids, and (4) the inhibitors did not contain lipids or heme.

Table 3. Compositions of purified lipase inhibitor I and II

Components	Contents(%)	
	I	II
Protein	47.50	19.25
Sugar	42.60	75.00
Lipid	—	—
RNA	6.28	3.64
DNA	0.52	0.33
Heme	—	—

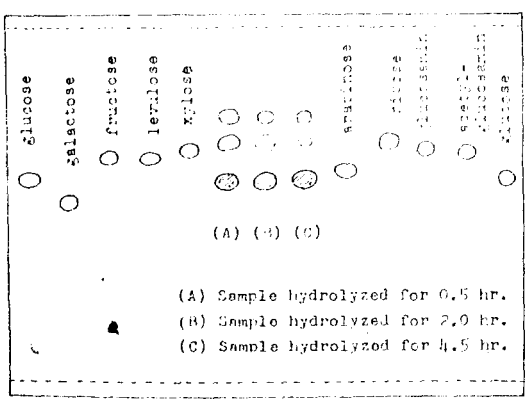

Fig. 7. Migrating patterns of sugar on cellulose thin layer chromatography

Stabilities of Inhibitory Activities of Purified Lipase Inhibitor I and II

It was found that there were the close relations between the chemical composition and the characters of purified lipase inhibitors.

The sugar components of inhibitors might be closely related to the stabilities of inhibitors. The purified lipase inhibitor II which contained a large amount (75%) of sugar was stable to a 30 min incubation at 100°C and at a broad pH range from 3 to 10 and also to the attacks of enzymes such as pronase, nagarse, glucoamylase, and RNase, wherease the purified lipase

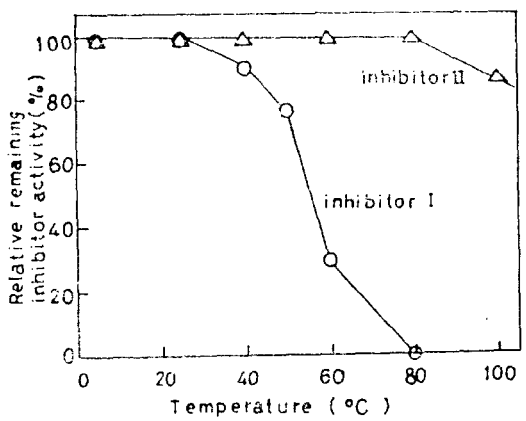

Fig. 8. Thermal stabilities of lipase inhibitors. The inhibitors were incubated in 0.01 M phosphate buffer of pH 7 for 30 min.

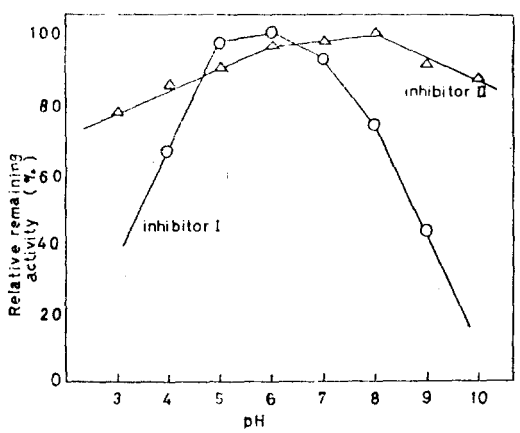

Fig. 9. Fig. 9. The pH stabilities of lipase inhibitors. The inhibitors were kept for 24 hours at the various pH conditions.

Table 4. Effects of enzymes on inhibitor activity. The inhibitors were incubated 4 hours at pH 7.0 and 37°C with 1/50 amounts of enzymes such as nagarse, pronase, glucoamylase, and RNase, respectively.

Items \ Enzymes / Inhibitors		RNase		Glucoamylase		Nagarse		Pronase	
		I	II	I	II	I	II	I	II
Lipase	(L)	27.7	27.7	27.7	27.7	27.7		27.7	27.7
Lipase+Enzyme	(E)	27.6	27.6	28.1	28.1	27.9		28.8	28.8
Lipase+Inhibitor	(I)	24.1	13.9	24.3	11.1	15.8		26.6	12.7
Lipase+Enzyme+Inhibitor	(C)	26.8	14.1	25.5	12.5	15.0		27.7	13.5
Remanining percent of inhibibitor activity		22.2	97.8	76.5	94.0	108.4		100.1	106.7

cf. Remaining percent(%) $= \frac{(L+C)-(L-E)}{(L-I)} \times 100$

Table 5. Effects of protein-degenerating reagents on purified lipase inhibitor II. The inhibitor was treated separately with 8M urea and 6M guanidine hydrochloride for 4 hours at room temperature.

Treatments	Lipase (Enzyme units)	Lipase+Inhibitor (Enzyme units)	Inhibition (%)
None	1.160	0.735	36.6 (100.0)
GuanidineHCl	1.160	0.925	20.3 (55.5)
Urea	1.160	0.870	25.0 (68.3)

inhibitor I which contained a relatively smaller amount (42%) of sugar was unstable to a 30 min incubation over 50°C and at the pH range below 5 or over 7 (Fig. 8 and 9).

Furthermore, the purified lipase inhibitor I was considerably inactivated by RNase or glucoamylase (Table 4).

This means that the sugar and RNA moieties contribute to the maintaining of activity of inhibitor I.

The inhibitory activity of purified lipase inhibitor II also decreased by the treatment with protein-denaturating agents such as urea and guanidine hydrochloride (Table 5).

This led to an assumption that the protein component was also related to the inhibitory activity of inhibitor. It seems unreasonable to assume that each single component, protein, sugar, and RNA, is independently related to the activity of inhibitor, because either component separated by some techniques such as isoelectrofocusing, Dowex 50×2 chromatography, and the extraction with some acidic solvents did not exhibit inhibitory activity.

Consequently, it was concluded that the inhibitory activities of purified lipase inhibitors were resulted from the cooperative action of the three components, protein, sugar, and RNA.

The Mode of Inhibition of Purified Lipase Inhibitor II

The purified lipase inhibitor II inhibited similarly the activity of pancreatic lipase toward various substrates such as methyl butyrate, tributyrin, trilaurin, and olive oil, as well as the activity of ordinary esterase toward isoamyl butyrate, but not the activities of nagarse, glucoamylase, and RNase.

The similar phenomena were reported by Satouchi *et al.* (1974) who observed the effects of inhibitor on various substrates such as soybean oil, olive oil, and ediol, and those on the activities of various enzymes such as lipases from various sources, amylase, and RNase.

Therefore, it was concluded that the purified lipase inhibitors were specific to the ester hydrolyzing enzymes, particularly to lipase.

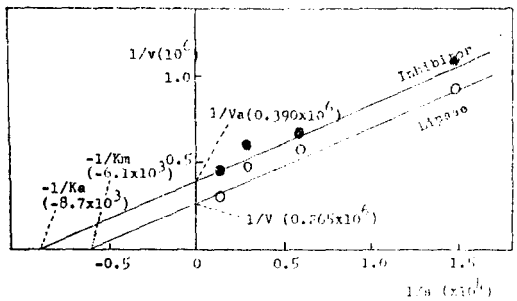

Fig. 10. Lineweaver-Burk plot of substrate concentration against reaction velocity.
The reaction mixtures contained 3.33×10^{-8} moles of purified lipsse inhibitor II, 5×10^{-4} moles of $CaCl_2$, 5×10^{-2} moles of KCl, and 0.336×10^{-4}, 1.683×10^{-4}, and 6.733×10^{-4} moles of tributyrin,respectively.

On the other hand, Lineweaver-Burk plot (Fig. 10) of the inhibition of purified lipase inhibitor II revealed that the type of inhibition was uncompetitive. This means that the inhibitor did not directly re actwith the active site of enzyme.

Concerning this problem, Satoudhi *et al.* (1974) reported that the lipase activity was inhibited immediately after the addition of inhibitor which did not cause the significant destruction of substrate emulsion, and the inhibitory activity was more effective when the inhibitor was preincubated with substrate than preincubated with lipase.

On the contrary, Vidal and Stoppani(1971) reported that the inhibition of phospholipase by inhibitor did not occur at least in full extent, unless the enzyme was incubated with inhibitor before the addition of lipoprotein substrate.

In the present experiment, the inhibition occured to the same extent in either case of the preincubation of inhibitor with lipase or that of inhibitor with substrate.

This finding implies that the purified lipase inhibitor slantingly reacts neither with enzyme nor substrate, and probably with enzyme-substrate complex (ES-complex), although there are no information to explain the interaction between the inhibitor and ES-complex.

Therefore, it might be supposed that the inhibition of lipase activity by the purified lipase inhibitor was not due to the reaction of inhibitor with the enzyme but the speific influences of inhibitor on the interfacial state of substrate based on the interaction between the inhibitor and ES-complex.

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