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

I. Separation and Some Properties of Crude Lipase Inhibitor

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A crude preparation of lipase inhibitors was separated from green pepper by extracting with a 0.8M NaCl solution, followed by saturating with ammonium sulfate to over 60%. The preparation was stable at the temperatures below 50°C for 30 min and at pH range from 3 to 10, and inhibited both pancreatic and rice bran lipases whereas the mode of these inhibition seems to be different. The preparation was much more effective on pancreatic lipase compared with rice bran lipase. In a very low concentration, the preparation showed accelerating effects on pancreatic lipase activity.

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 substances that are currently used (Pressey, 1972).

As the natural lipase inhibitors, Brandle (1970) separated protease from microbes or vegetables, Hochstraßer *et al.* (1971) lipid from seeds of ***Arachis hypo-***

gaea, Milic et *al. (1972)* tannins from lucerne, and Satouchi et *al. (1974)* inhibitor-protein from soybean seeds.

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

In the present paper, the separation methods and some properties of a preparation of crude inhibitors from green pepper will be introduced.

MATERIALS AND METHODS

1. Materials

(1) Green pepper

The samples used in this experiment were green pepper *(Capsicum annuun* Lin., Cultivar Shinsakigake) which were harvested at Miyazaki Prefecture, Japan, in April, 1975. In this experiment, the unripe green pepper was excluded because of smaller contents of inhibitors compared with the mature (Table 1).

Table 1. Contents of inhibitor under different growth phases.

Growth phase	Total OD (at 280 nm)	Spec. Act. ($1/OD_{280}$ at I_{50})	Total Act. (per kg of sample)
Unripe	3750.7	0.506	1898.3
Mature	3616.7	0.630	2278.5

(2) *Tributyrin*

As a substrate of lipase, tributyrin (a product of Nakarai Chemical, Ltd.) was used.

(3) *Pancreatic lipase*

Stearin purchased from Tokyo Kasei Co. was used. This preparation contained about *60* units of lipase per mg.

(4) *Rice bran lipase*

Rice bran lipase was contributed from Dr. Aizono in our laboratory.

2. Methods

(I) *Extraction of crude inhibitors*

In order to separate inhibitor, green pepper (50 kg) was cleansed, removed from sheaths and then homogenized in an ice cold 0.85M NaCl solution (50 l) which was the effective solvent as shown in Fig. 1.

After homogenation, inhibitors were extracted over night in a low temperature room (about 7°C) without stirring, because some oxidative degradation or denaturation of inhibitor might be taken place by stirring (Table 2).

The extracts were strained through cheese cloths, followed by a centrifugation at *6000* rpm for 10 min using a refrigerative centrifuge (5°C). The resulting supernatant was used as a crude extract for the separation of inhibitor.

(2) *Separation of crude inhibitor preparation*

The crude extract was saturated with ammonium sulfate to 0.6 and centri-

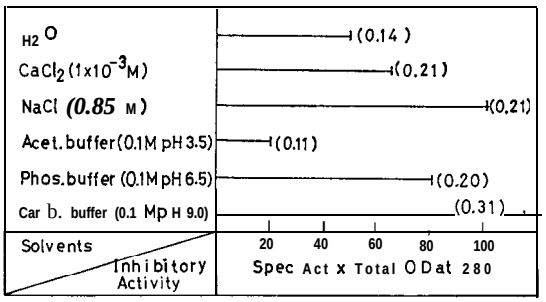

Fig. **1.** Extraction of inhibitor with various solvents. Each 100 g of green pepper was homogenized in 100 ml of various ice cold solvents described in figure and extracted over night in a low temperature room (about 7°C). The parenthesized numbers in figure represent specific activities of inhibitor extracted by the corresponding solvent.

Table 2. Effects of stirring on extraction of inhibitor.

Samples	Total OD (at 280 nm)	Spec. Act. ($1/OD_{280}$ at I_{50})	Total Act. (per kg of sample)
Stirred	3619.5	0.620	2244.1
Nonstirred	4018.7	0.635	2549.8

fuged at 8000 rpm and 5°C for 20 min. The resulting supernatant was brought to saturation with ammonium sulfate and centrifuged at 8000 rpm and 5°C for 20 min.

The inhibitors were yielded in the resulting precipitate by about 90 % (Table 3). The precipitate was dissolved in a small amount of deionized water and dialyzed against deionized water using Visking tube No. 30/32. The dialyzed solution were centrifuged to remove any insoluble materials and lyophilized.

The lyophilized products were employed throughout this experiment as a crude inhibitor preparation.

(3) ***Assay of lipase***

In this experiment, the assay of pancreatic lipase was carried out as described by Funatsu et al. (1971). A mixture of 1.0 ml of 0.5M KCl, 1.0 ml of 5×10^{-3}M $CaCl_2$ and a given amount of the enzyme was made up the volume to 9.8 ml with deionized water, incubated for 10 min at 35°C and then carried out the enzyme reaction at pH 7.5 on the addition of 0.2 ml of tributyrin.

The free fatty acid produced in consequence of the enzyme reaction was determined with 0.025 or 0.1N NaOH using a pH stat (Radiometer Type TTT-1C).

The enzyme unit was expressed as one unit as the amount of enzyme that hydrolyzed one micromole of ester bond in the substrate per minute.

(4) ***Assay of inhibitor***

The assay of inhibitor was followed by estimating the activity of pancreatic lipase remained after incubating the reaction mixture with inhibitor, that is, a given amount of inhibitor was added to the enzyme solution containing 1.0

ml of 0.5M KCl, 1.0 ml of 5x 10^{-3}M $CaCl_2$ and 1.5 units of enzyme. The total volume was made up to 9.8 ml with deionized water. After an incubation at 35" C for 10 min, the remaining lipase activity was estimated using 0.2 ml of tributyrin as a substrate.

The activity of inhibitor was expressed as a reciprocal of the protein concentration (OD at 280 nm) of the inhibitor that inhibited 50 % of pancreatic lipase (1.5 units).

RESULTS

1. Fractionation of the crude extracts with ammonium sulfate

The crude extract was saturated with ammonium sulfate to 0.2 and the resulting precipitate (0.2 fraction) was separated by a centrifugation at 8000 rpm for 10 min. The supernatant was saturated again with ammonium sulfate to 0.4 and the precipitate (0.4 fraction) was separated by a centrifugation at 8000 rpm for 10 min. In such a way, 0.6, 0.8, and 1.0 fractions were separated succeedingly. The activity of inhibitor contained in each fraction was estimated as shown in Table 3.

Table 3. Fractionation of crude extracts with ammonium sulfate.

Fractions	Total OD (at 280 nm)	Spec. Act. ($1/OD_{280}$ at I_{50})	Total Act. (per kg of sample)
0.2			5.7
0.6	82.6 23.6	0.4813	61..3
0.8	1524.6 204.4	0.829	1263.9 166.2
1:o	1489.6	0: 385	573.5
Supernatant	1549.2	0.013	20.1

As shown in Table 3, about 90 % of lipase inhibitors were contained in the fractions between 0.6 and 1.0.

2. Yield of crude inhibitor from green pepper

The yield of crude inhibitor obtained from green pepper is shown in Table 4.

Table 4. Yield of crude inhibitor.

Items	Total OD (at 280 nm)	Spec. Act. ($1/OD_{280}$ at I_{50})	Total Act. (per kg of sample)
Crude extract	9177.0	0.250	2294.3
Crude inhibitor	1502.2	0.901	1353.5

3. Thermal and pH-stabilities of crude inhibitor

To characterize the crude inhibitor the thermal and pH-stabilities of the preparation were examined.

(1) Thermal stability of the crude inhibitor

To each 1 ml of crude inhibitor solution was added 2 ml of 0.01M phosphate buffer, pH 7 and incubated for 30 min at 0, 15, 25, 40, 50, 60, and 80°C, respectively. After removing the precipitates, if any, the remaining inhibitor in supernatant was assayed. The results are shown in Fig. 2.

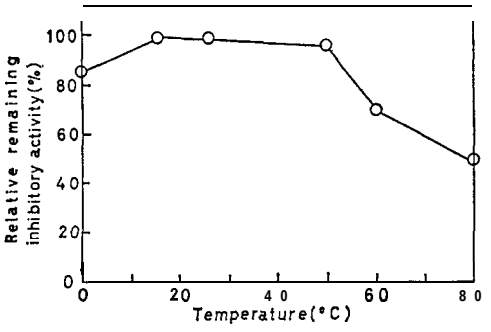

Fig. 2. Thermal stability of crude inhibitor.

As shown in Fig. 2, the crude inhibitor was stable at the temperatures from 0 to 50°C for 30 min, whereas the inhibitory activity was lowered by about 50% at 80°C.

(2) *pH-stability of the crude inhibitor*

Each 1 ml of crude inhibitor solution was adjusted to pH 3, 4 (with 2 ml of 0.01M acetate buffer), 5, 6, 7 (with 2 ml of 0.01M phosphate buffer), 8, 9, and 10 (with 2 ml of 0.01M carbonate buffer) and kept for 24 hr at 5°C. After incubation, each solution was dialyzed separately against deionized water for 36 hr. The remaining inhibitory activity was measured. The results are shown in Fig. 3.

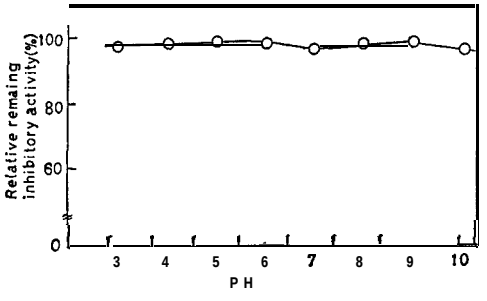

Fig. 3. p&stability of crude inhibitor.

As shown in Fig. 3, the crude inhibitor was stable at a pH range from 3 to 10.

4. Relation between the concentration and the inhibitory activity of the crude inhibibitor preparation

The inhibitory activities of the crude inhibitor preparation toward pancreatic and rice bran lipases were estimated at various concentrations of inhibitor. The results are shown in Fig. 4.

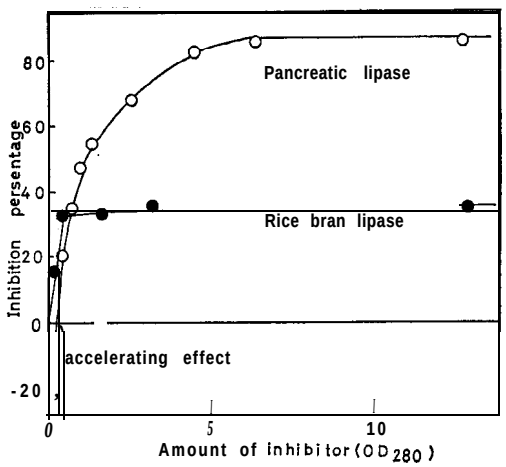

Fig. 4. Relation between inhibitory activity and amount of inhibitor. *To* each enzyme solution containing 1.0 ml of 0.5M KCI, 1.0 ml of 5×10^{-3}M $CaCl_2$ and 1.5 units of pancreatic lipase or 0.5 unit of rice bran lipase, the various amounts of inhibitor were added and made up the total volume to 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.

As shown in Fig. 4, the inhibitory activity toward pancreatic lipase was generally greater than those toward rice bran lipase. It was particularly of interest that the preparation showed an accelerating effect on pancreatic lipase at the concentrations below 0.4 of OD at 280 nm.

DISCUSSION

Most of lipase inhibitor in the extract of green pepper came to the fraction precipitated with over 0.6 saturation of ammonium sulfate. The separated lipase inhibitor was dissolved in water as well as in salt solutions. It was supposed that the lipase inhibitor was a kind of albuminous materials.

Machovich *et al.* (1970) purified partially a rat pancreas lipase inhibitory factor from a rat liver homogenate and established that the inhibitor was associated with protein having a molecular weight of 100,000 and its inhibitory activity was abolished by the treatment with trypsin, but not with RNase. By heat treatment at 50 and 60°C for 5 min, 50 and 100 % of its inhibitory activity were destroyed, respectively.

Mori *et al.* (1973) reported that the inhibitor preparation from soybean seeds was fractionated by ammonium sulfate saturation, between 0.25 and 0.5. The preparation was inactivated by heat or pronase treatments.

However, the activity of lipase inhibitor preparation from green pepper remained 95 and 50 % of the original by heat treatment at 50 and 80°C for 10 min, respectively (Fig. 2).

In comparison with these reports, it was considered that the inhibitor in this experiment was different from those described above, and that it was a protein complex containing certain stable components besides protein. It needs, however, much more studies to elucidate the active compositions of the inhibitors.

The inhibitory activity toward pancreatic and rice bran lipases seems to suggest that the inhibitor was lack in the source specificity even though its inhibition mode was characteristically represented in connection with the mode of corresponding enzyme action. This phenomenon was agreed with the reports of many investigators (Bowman, 1945; Ryan, 1966 ; Satouchi et al., 1974) that the natural enzyme inhibitors have generally the unusual properties of being absent of specificity for a single enzyme and species-specificity for plant enzyme.

In addition, it was also of interest that the crude preparation inhibited pancreatic lipase at a high concentration, but accelerated at a very low concentration. The research on this problem is on progress.

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