Purification and Properties of a Lipase Inhibiting Protein from Soybean Cotyledons

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Received October 23, 1975

A proteinous inhibitor of lipase was purified from soybean cotyledons by the procedures of ammonium sulfate fractionation, gel-filtration on Sephadex G-150 column, DEAE-cellulose column chromatography and isoelectric focusing. The inhibitor obtained by gel-filtration was separated into three active components, D-1, D-2 and D-3, by DEAE-cellulose column chromatography. The D-3 fraction after isoelectric focusing was homogeneous judged from disc electrophoresis. The inhibitory activity was more stable against treatments of heating and Pronase when the D-3 fraction was preincubated with substrate than without substrate. The extent of inhibition was varied by changing the order of addition of reactants and condition of substrate. From these results, the mode of inhibition is discussed.

The hydrolysis of all lipolytic enzymes, including lipase, occurs at the interface between water and oil, and these enzymes hydrolyze water-insoluble substrate at the maximum rate only when an adequate interfacial area is maintained. Therefore, any substance increasing or decreasing the interfacial area of the substrate may act apparently as an activator or inhibitor of these enzymes.1,2 In the study of a factor changing the activities of lipolytic enzymes, it is necessary to elucidate whether the activation or inhibition is caused by the enzyme or substrate.

Mattson et al.,3 have reported that the hydrolysis of methyloleate by the action of pancreatic lipase is inhibited by the addition of normal alcohols to the substrate, and this effect is due to the adsorption of alcohols on the substrate, thus, the substrate is blocked from the enzyme attack. Brockerhoff4 has reported that albumin added to the substrate at a high concentration blocks the interface of the substrate and reduces the rate of lipolysis.

Sugiura et al. have described an other inhibitor that phospholipids inhibit the activity of lipase from C. paralipolytica,5 but a proteinous inhibitor of lipase has not been found except an inhibitor protein from rat liver for rat pancreatic lipase.6

Recently, the authors have reported that a protein from soybean seed extract inhibits the hydrolysis of soybean oil emulsion by pancreatic lipase.7,8 In this paper, purification of this protein and the mode of inhibition is described.

MATERIALS AND METHODS

Materials. Purified porcine pancreatic lipase was purchased from Calbiochem. Bovine serum albumin, γ-globulin, ovalbumin and gum arabic were purchased from Sigma Chem. Co. Pronase was purchased from Kaken Chemicals. Carrier ampholytes (pH 5 – 7) were obtained from LKB Producer AB. Sephadex G-150 and G-200 were the products of Pharmacia Co. DEAE-cellulose was the product of Brown Co.

Preparation of the substrates. Soybean oil emulsion was prepared as follows: One ml of soybean oil was added to 10 ml of 5% gum arabic (dialyzed overnight against water) in a 30 ml beaker and the mixture was stirred at about 1,000 rpm with a magnetic stirrer for 5 hr or dispersed with a sonicator, Kaijo Denki T-A-4201, at 20 KC for 5 min in an ice bath. The substrates, triacetin (10%), tripropionin (1%) and tributyrin (0.25%) were dispersed in 100 ml of 0.1 m aqueous NaCl by vigorous stirring at about 1000 rpm.

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with a magnetic stirrer for 30 min.

Assay of lipase activity. 1. The hydrolysis of soybean oil was assayed by measuring free fatty acids liberated in the reaction mixture by the method of Duncombe9) using linoleic acid as the standard. The reaction mixture was similar to that described in the previous paper9) with the exception of using potassium phosphate buffer (pH 7.0), 100 µl of soybean oil emulsion and 10 µg of purified pancreatic lipase. The reaction was started by the addition of substrate and incubation was performed at 37 or 30°C for 10 or 20 min as described in figures and tables.

2. The initial hydrolysis of short-chain triglyceride was measured titrimetrically with a Radiometer pH-stat at 25°C and pH 7.5 under nitrogen gas stream on 3 ml of substrate in 1 mm calcium acetate. The reaction was started by the addition of lipase to the substrate, and stirring was maintained after the addition of lipase. The flow rate of 0.01 NaOH was recorded as a function of time.

Assay of inhibitory activity. To determine the inhibitory activity in a given sample, a sample solution was preincubated with lipase solution for a few minutes at room temperature. The substrate emulsion was added and the remaining activity of lipase was determined. This order of adding enzyme, inhibitor and substrate was used for a routine assay of inhibitory activity. The inhibitor unit was defined as the amount of inhibitor which decreased the activity of 10 µg of lipase to 50%.

Isoelectric focusing. Isoelectric focusing experiments were carried out at 4°C on a column of 30 ml capacity as described by Doi and Ohtsuru.10) The carrier ampholytes covering the pH range between 5 to 7 was used at an average final concentration of 2.5%. After focusing was carried out for 48 hr at 800 V, fractions of 1 ml were collected from the bottom of the column. The amount of protein in each fraction was determined spectrophotometrically at 280 nm, and the pH of each fraction was measured by a pH meter with a combined electrode.

Polyacrylamide gel disc electrophoresis. Disc electrophoresis was performed in polyacrylamide gel as described by Ornstein.11) The electrophoresis was carried out for about 4 hr at 250 V with a current of 2.0 mA/tube using Tris-glycine buffer (pH 8.3). Protein band in the gel was detected by staining with Coomassie blue, and destaining with 7% (v/v) acetic acid.

Protein determination. Protein concentration was determined by the method of Lowry et al.12) using bovine serum albumin as the standard. The protein profiles of column chromatography were followed by measuring the absorbances of respective fractions at 280 nm.

RESULTS

Purification of the inhibitor

The purification procedures were carried out at 4°C and centrifugations were performed at 10,000 × g for 30 min in a refrigerated centrifuge unless otherwise mentioned.

Step 1. Dry cotyledons of soybean seeds (20 g) were ground with a Willey mill and homogenized in 120 ml of 0.1 M potassium phosphate buffer (pH 7.0) containing 0.5 M 2-mercaptoethanol. After filtration through four sheets of gauze, the filtrate was centrifuged.

Step 2. To the resulting supernatant, solid ammonium sulfate was added to give 30% saturation. The solution was stirred for 15 min and then allowed to stand for 45 min. The precipitate was removed by centrifugation. The supernatant was brought to 50% saturation of ammonium sulfate, stirred for 15 min, allowed to stand for 45 min and centrifuged. The precipitate which contained 87% of the total inhibitory activity was dissolved in a minimum volume of 0.1 M potassium phosphate buffer (pH 7.0) containing 0.5 M KCl and 0.5 M 2-mercaptoethanol.

Step 3. The resulting solution was placed on the top of a column of Sephadex G-150 (2.5 × 90 cm) which had been previously equilibrated with 0.1 M potassium phosphate buffer (pH 7.0) containing 0.5 M KCl and 0.5 M 2-mercaptoethanol. Elution was carried out with the same buffer at a flow rate of 15 ml/hr and 5 ml were collected for each fraction. The inhibitory activity of each fraction was assayed. Two peaks of the inhibitory activity appeared as shown in Fig. 1. The major peak, fraction No. from 55 to 69, was pooled. To this solution, solid ammonium sulfate was added to give about 60% saturation. The solution was allowed to stand for 1 hr and centrifuged. The precipitate was dissolved in a
small volume of 0.01 M potassium phosphate buffer (pH 7.0) containing 0.5 mM 2-mercaptoethanol. The solution was dialyzed overnight against the same buffer, and the insoluble material formed during dialysis was removed by centrifugation.

**Step 4.** The dialyzed solution was applied to a DEAE-cellulose column (2.5 × 40 cm) which had been previously equilibrated with 0.01 M potassium phosphate buffer (pH 7.0) containing 0.5 mM 2-mercaptoethanol. The unadsorbed materials were washed out through the column with the same buffer. The inhibitor was eluted from the column with linear gradient of KCl from 0 to 0.4 M at a flow rate of 30 ml/hr. As shown in Fig. 2, three peaks of the inhibitory activity appeared. Those peaks were designated as D-1, D-2 and D-3 as indicated in the figure. The inhibitory activity of the D-3 was completely separated from other two peaks and the D-3 fraction revealed to be of high specific activity. Then, the purification and characterization of D-3 fraction was attempted. The D-3 fraction, fraction No. from 65 to 77, was pooled. To the combined fraction, solid ammonium sulfate was added to give about 60% saturation. The solution was allowed to stand for 1 hr, and the precipitate was collected by centrifugation. This was dissolved in a small volume of 0.01 M potassium phosphate buffer (pH 7.0) containing 2-mercaptoethanol and dialyzed overnight against the same buffer.

**Step 5.** Part of the dialyzed solution was then subjected to the isoelectric focusing on a column of 30 ml capacity with a density gradient of sucrose from 0 to 70% and the pH gradient of pH 5 to 7. The result is shown in Fig. 3. The fraction with inhibitory activity was dialyzed for 24 hr against the same buffer used in the step 4 to remove carrier ampholyte and sucrose. An aliquot of the dialyzed inhibitor preparation was subjected to polyacrylamide gel electrophoresis to see the purity of the preparation. As shown in Fig. 4, the inhibitor preparation obtained after isoelectric focusing gave a single protein band.

By these procedures up to step 4, the specific activity of D-3 fraction increased 12.6 fold and the recovery was 4.8% of the original activity.
FIG. 3. Isoelectric Focusing of the D-3 Fraction Obtained from DEAE-Cellulose Column Chromatography.

For the determination of the inhibitory activity, 100 µl of each fraction was used. The assay condition was the same as described in Fig. 1. The dense anode solution was composed of 0.2 ml of phosphoric acid and 20 ml of 70% (w/v) sucrose aqueous solution and light cathode solution was composed of 0.4 ml of ethylene diamine and 10 ml of water. A linear gradient of sucrose from 0% to 70% was prepared in the focusing column (30 ml) by using Mitsumi Gradienter. About 4 mg of D-3 fraction was used for this experiment.

--- absorbance at 280 nm; •-•, inhibitory activity; ○—○, pH.

Properties of D-3 fraction protein

The D-3 fraction after isoelectric focusing, dissolved in 0.01 M potassium phosphate buffer (pH 7.0) containing 0.5 mM 2-mercaptoethanol, showed a maximum absorption near 280 nm.

The isoelectric point of the D-3 fraction was determined to be pH 5.75 from the effluent pattern shown in Fig. 3.

The molecular weight of the D-3 fraction was estimated by gel-filtration on Sephadex G-200 according to the procedure of Andrews. Ovoalbumin, bovine serum albumin and γ-globulin were used as the standard proteins, and the elution volume of D-3 fraction, determined by assaying the inhibitory activity, corresponded to a molecular weight of 77,000.

Stability of the D-3 fraction

pH. The D-3 fraction was incubated at 20°C for 90 min at various pHs using the following buffer solution; 0.05 M acetate buffer for pH 4.5 to 6.0, 0.05 M phosphate buffer for pH 6.0 to 8.0 and 0.05 M Tris buffer for pH 8.0 to 9.5. As shown in Fig. 5, the inhibitory activity of the D-3 fraction was stable over a wide pH range, with a peak at pH 6.0.

FIG. 5. Effect of pH on Stability of the D-3 Fraction.

The D-3 fraction, dissolved in each buffer as described below, was preincubated at 20°C for 90 min, and the remaining inhibitory activity was measured under the condition as described in Fig. 1. The final amount of the D-3 fraction used was 40 µg.

---, potassium-acetate buffer; •—•, potassium-phosphate buffer; ○—○, Tris-HCl buffer.

Fig. 4. Polyacrylamide Gel Electrophoresis of the D-3 Fraction before and after Isoelectric Focusing. (1) D-3 fraction after isoelectric focusing, (2) D-3 fraction.
activity was stable in the pH range of 6.5 to 9.5.

Temperature. The D-3 fraction, dissolved in 0.1 M phosphate buffer (pH 7.0), was incubated with or without soybean oil emulsion at various temperatures for 10 min and the remaining inhibitory activity was determined. As shown in Fig. 6, the D-3 fraction was stable up to 33°C and lost its activity almost completely above 50°C, when incubated without substrate emulsion. However, the addition of soybean oil emulsion to the heating mixture effected to raise its thermal stability, and the inhibitory activity was still stable at 60°C. Above this temperature, the control lipase activity decreased linearly because of the destruction of soybean oil emulsion. The inhibitory activity also decreased.

Pronase. The D-3 fraction, dissolved in 0.1 M phosphate buffer (pH 7.8), was treated with Pronase at 30°C for various times with or without substrate soybean oil emulsion, and the remaining inhibitory activity of the D-3 fraction was determined. As shown in Fig. 7, the inhibitory activity of the D-3 fraction was completely lost by preincubation with Pronase for 60 min, but in the presence of soybean oil emulsion, the activity was hardly affected by preincubating with Pronase for 120 min.

Mode of inhibition
The effect of the sequence of addition of reactants on the extent of inhibition was examined according to the following procedures. (I) D-3 fraction was added to the reaction mixture where the hydrolysis of soybean oil emulsion by lipase had been occurring. (II) D-3 fraction and lipase were preincubated for 5 min, and then the reaction was started by the addition of substrate. (III) D-3 fraction and substrate were preincubated for 5 min, and then the reaction was started by the addition of lipase. As shown in Fig. 8, inhibitory activity
FIG. 8. Effect of the Order of the Addition of Reactants on Inhibitory Activity.
Reactants, inhibitor (I), soybean oil emulsion (S) and lipase (L), were added at the different order. (I) (L+S)+I, (II) (I+L)+S, (III) (I+S)+L. Inhibitory activity was measured in 0.1 M potassium phosphate buffer (pH 7.8) at 30°C by using 50 µg of the D-3 fraction.

0-0, no inhibitor.

was more effective in the procedure of (III) than in the procedures of (I) and (II).

Effect of condition of the substrate
Short-chain triglycerides. Inhibitory activity of the D-3 fraction on the hydrolysis of short-chain triglycerides was examined by using tributyrin, tripropionin and triacetin emulsions as the substrate according to the procedures as mentioned above. Inhibitory activity was more effective in the procedure of (III) than in the procedures of (I) and (II), when tributyrin or tripropionin was used as the substrate, but when triacetin was used as the substrate, inhibitory activity was lower than when tributyrin and tripropionin were used as the substrate, even if the inhibition was examined according to the procedure of (III) (Table I).

Concentration of the substrate. The inhibitory activity of the D-3 fraction with various amount of soybean oil emulsion, at the range from 25 µl to 200 µl in total volume of 500 µl, was examined according to the procedures of (II) and (III) as mentioned above. Inhibition in the procedure of (II) decreased steeply at the higher amount of the substrate than 100 µl, and Lineweaver and Burk plot in the procedure of (III) was of competitive type (Fig. 9).

Sonicated emulsion. Lipase activity did not depend upon the amount of the substrate but the available interfacial area of the sub-

TABLE I. INHIBITION OF THE D-3 FRACTION ON THE HYDROLYSIS OF SHORT-CHAIN TRIGLYCERIDES BY LIPASE

In the case of substrate of tributyrin (0.25%) and tripropionin (1.00%), inhibitory activity of the D-3 fraction (50 µg) against lipase (2 µg) were measured for 2.5 min and in the case of substrate of triacetin (10.0%), inhibitory activity of the D-3 fraction (250 µg) against lipase (10 µg) was measured for 2.5 min. The experimental details are described in the text.

<table>
<thead>
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<th>Substrate</th>
<th>Order of adding inhibitor</th>
<th>0.05</th>
<th>0.03</th>
<th>0.02</th>
<th>0.01</th>
<th>0.009</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tributyrin (0.25%)</td>
<td>II</td>
<td>600</td>
<td>165</td>
<td>215</td>
<td>335</td>
<td></td>
</tr>
<tr>
<td>Tripropionin (1.0%)</td>
<td>III</td>
<td>450</td>
<td>215</td>
<td>225</td>
<td>285</td>
<td></td>
</tr>
<tr>
<td>Triacetin (10.0%)</td>
<td>I</td>
<td>185</td>
<td>140</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
</tbody>
</table>

FIG. 9. Effect of the Concentration of Soybean Oil Emulsion on Inhibitory Activity.
Inhibitory activity of the D-3 fraction (50 µg) with various amount of soybean oil emulsion (prepared by vigorous stirring) were examined in 0.1 M potassium phosphate buffer (pH 7.8) at 30°C for 10 min according to the procedures of (II) and (III) as described in Fig. 8.

0-0, no inhibitor.
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FIG. 10. Inhibition of the D-3 Fraction on Hydrolysis of Sonicated Soybean Oil Emulsion by Lipase.

Inhibitory activity of the D-3 fraction was examined in 0.1 M potassium phosphate buffer (pH 7.8) at 30°C for 10 min according to the procedures of (III) by using 100 µl of soybean oil emulsion prepared by sonication or stirring.

-○-, sonicated emulsion; ● - ●, stirred emulsion.

Then, in order to obtain more dispersed emulsion, soybean oil was emulsified into a solution containing 5% of gum arabic with a sonicator. As shown in Fig. 10, the action of lipase was more active when sonicated emulsion was used as the substrate than when stirred emulsion was used. But the inhibition by the action of the D-3 fraction, which was observed when stirred emulsion was used as the substrate, was not observed when sonicated emulsion was used as the substrate even if an excess amount of inhibitor was added.

Addition of α-tocopherols. α-Tocopherol or α-tocopherol acetate was added to the sonicated emulsion by vigorous stirring, and the inhibitory activity of the D-3 fraction was examined by using this emulsion as the substrate. As shown in Table II, α-tocopherol or α-tocopherol acetate itself was inhibitory, and the additional inhibitory activity of the D-3 fraction was observed when this emulsion was used as the substrate.

Concentration of sonicated emulsion. When 100 µl of sonicated emulsion was used as the substrate, inhibition, as was observed when the same amount of stirred emulsion was used, was not observed. Then, the inhibitory activity of the D-3 fraction was examined at lower amount of sonicated emulsion than 100 µl. As shown in Table III, the smaller the sonicated emulsion was used, the larger the extent of inhibition was observed.

DISCUSSION

Lipase activity was increased by the addition of monovalent cations to the reaction mixture. This effect was characteristic when NaCl or LiCl was added to the reaction mix-
ture, but in the case of KCl, the increase of lipase activity was not so high as in the case of NaCl or LiCl, thus, potassium phosphate buffer and KCl were used in the experiments for purification to avoid the complexity of inhibitory activity.

The inhibitor preparation obtained by gel-filtration on Sephadex G-150 (Fig. 1) was separated into three components (D-1, D-2 and D-3) by DEAE-cellulose column chromatography (Fig. 2). The isoelectric focusing pattern of this inhibitor preparation also showed that it contained three components, and the isoelectric points of inhibitors were determined to be pH 5.73, 5.99 and 6.10, respectively (not shown). As shown in Fig. 3, the isoelectric point of the D-3 fraction was determined to be pH 5.75, and from the result of isoelectric focusing of D-1 fraction, its isoelectric point was determined to be pH 6.00 (not shown). Catsimpoolas and Meyer14) separated four different soybean hemagglutinin by using isoelectric focusing and their isoelectric points were determined to be pH 5.85, 6.00, 6.10 and 6.20, respectively. Soybean lipase inhibitors were similar to soybean hemagglutinin at the point of isoelectric point, but the molecular weight of soybean lipase inhibitor was different from that of soybean hemagglutinin, that is, the former was calculated to be 77,000 and the latter was 120,000 by gel-filtration.16)

As the D-3 fraction was not stable at lower pH than 6.5 (Fig. 5), the activity decreased during the experiment of isoelectric focusing. Therefore, the purified D-3 inhibitor which was isolated in disc electrophoretically homogeneous form by isoelectric focusing (Figs. 3 and 4) could not be used for the experiments of inhibitory properties.

In the experiment of thermal stability of the D-3 fraction, it lost almost completely the inhibitory activity above 50°C, but in the presence of soybean oil emulsion, the D-3 fraction was still stable at 60°C (Fig. 6). This stabilizing effect of soybean oil emulsion against heating was not due to the presence of gum arabic but of soybean oil or its emulsion form. The D-3 fraction was also inactivated completely by the digestion with Pronase at 30°C for 60 min, whereas in the presence of soybean oil emulsion, 82% of inhibitory activity was still preserved even if the pre-incubation time was prolonged to 120 min (Fig. 7). Lipase activity was not affected by the addition of 4 fold amounts of Pronase to the reaction mixture, and the proteolytic activity of Pronase was not also affected by the addition of soybean oil to the reaction mixture, that was examined by using casein as the substrate. These results show that the D-3 fraction has some affinity to the interface of substrate soybean oil emulsion.

Different procedures for the assay of inhibitory activity were examined by using soybean oil, tributyrin and tripropionin emulsions as the substrates (Fig. 8 and Table I). Inhibitory activity was more effective when the reaction was started by the addition of lipase to the mixture where inhibitor and substrate emulsion had been preincubated than when the reaction was started by the addition of substrate emulsion to the mixture where inhibitor and lipase had been preincubated. This result again shows that the inhibitory protein has an affinity to the substrate emulsion and probably can adsorb onto the interface of the substrate.

As shown in Fig. 9, the inhibition in the procedure (III) was of competitive type. Benzonana and Desnuelle16) showed that Lineweaver and Burk plots of lipolysis rate against substrate weight in a coarse and fine emulsions for the same number of lipase units was of competitive type. Thus, inhibition caused by the decrease of available interfacial area of substrate is kinetically indistinguishable from true competitive inhibition. But considering from the result that this inhibitory protein has some affinity to the substrate emulsion, the inhibition seems to be caused from the side of the substrate. This conclusion is supported by the result that the inhibition was not observed when soybean oil emulsion prepared by sonication was used as the substrate and observed again when α-tocopherol
was added to the sonicated emulsion and this mixture was used as the substrate (Fig. 10 and Table II). The cause that the inhibition was not observed when the sonicated emulsion was used as the substrate seems to be due to the increase of interfacial area of the substrate (Fig. 10). α-Tocopherol alone inhibited the hydrolysis of soybean oil emulsion by pancreatic lipase (Table II). As shown by Mattson et al., long-chain alcohol which contains partly hydrophobic aliphatic chain and partly hydrophilic hydroxy group inhibited the hydrolysis of methylolate by lipase because of its adsorption on the interface. Brockerhoff and Jensen have described in their review that the substance which has amphipathic character can distribute itself between water-oil interface and inhibit the lipase activity. Judging from the result that such amphipathic substance as long-chain alcohol inhibits the activity of lipase, α-tocopherol also seems to be adsorbed onto the interface of soybean oil emulsion and inhibits the lipase activity by the dilution of the substrate at interface. Thus, because of the decrease of the interfacial area of the substrate, the additional inhibition is observed by adding the D-3 fraction to the substrate which is prepared by stirring the mixture of sonicated emulsion and α-tocopherol vigorously. Therefore, the inhibition occurred by the addition of D-3 fraction to the reaction mixture for lipase activity causes not by the interaction between lipase and inhibitor but between the inhibitor and substrate emulsion. In other words, the degree of inhibition by the action of the D-3 inhibitory protein is determined not by the relationship between the amounts of lipase and the inhibitor but those of substrate emulsion (interfacial area) and the inhibitor.

REFERENCES

4) H. Brockerhoff, ibid., 246, 5828 (1971).