Purification and Characterization of Proteinous Inhibitor of Lipase from Wheat Flour

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Proteinous lipase inhibitor (LI) from wheat flour was purified to homogeneity using hydrophobic chromatography, gel chromatography, affinity chromatography, SDS-PAGE, and two-dimensional electrophoresis. Molecular masses of the LIs were approximately 28 and 25 kDa as estimated by SDS-PAGE. The amino acid sequences from the N termini of the three components detected by two-dimensional electrophoresis were Arg-Ser-Ala-His-Glu-Pro-Gln-Gln-Pro in the two 25 kDa isolates and Arg-Ser-Ala-His-Glu-Glu-Gln-Gln-His in the 28 kDa isolate, and they were suggested to be genetic variants. The LI was activated by the heating procedure up to 80 °C and stable in the pH range from 3.0 to 7.0. Porcine pancreatic lipase was inhibited through direct interaction with LI, while the activity fo the lipase from *Candida cylindracea* was partially inhibited and those of the other microbacteria were not inhibited.

Keywords: *Lipase inhibitor; wheat flour; pancreatic lipase*

INTRODUCTION

Many enzyme inhibitors are widely distributed in plant seed. Soybean trypsin inhibitor and wheat α-amylase inhibitors have been extensively studied, and they have been called the α-amylase/trypsin inhibitor family due to their high degree of homology (Kashlan and Richardson, 1981; Barber *et al.*, 1986).

On the other hand, the properties of lipase inhibitor (LI) in plant and the mechanism of inhibition have not been well elucidated, because the hydrolysis of lipase occurs at the interface between water and oil. Satouchi *et al.* (1974) and Satouchi and Matsushita (1976) reported the purification of a lipase protein inhibitor from soybean seeds, and from kinetic studies it was suggested that inactivation of pancreatic or microbial lipases was caused not by the direct interaction between enzyme and inhibitor but rather by the interaction between inhibitor and emulsified lipid substrate. From *in vitro* studies, wheat bran and wheat germ have been also reported to inhibit the activity of pancreatic lipase, the key enzyme for fat digestion, and proteins inhibiting this lipase have been isolated from their sources, with relative molecular masses ranging from 24 400 to 27 500 (Borel *et al.*, 1989). Inhibition of pancreatic lipase by the wheat germ proteins is also related to their ability to interact with the emulsified substrate and to hinder the adsorption of the enzyme on the interface, and recently the inhibition of pancreatic lipase activity by extracts of raw, milled, or processed wheat and other cereals was measured *in vitro* (Cara *et al.*, 1992). It was shown that milling of whole grain into flours markedly decreased the lipase inhibitory activity, and the germ and aleuron-layer fraction in durum wheat exhibited the highest inhibitory activities.

Thus, we have found it of interest to investigate further the inhibitory effect of proteins on lipase activity in wheat flour. In the present study, we isolated proteinous lipase inhibitor from wheat flour, which indicated the lipase inhibitory effect by direct interaction between enzyme and inhibitor.

MATERIALS AND METHODS

Chemicals. Lyophilized porcine pancreatic lipase (type VI-s) and lipases of *Candida cylindracea*, *Rhizopus arrhizus* (type XI), *Chromobacterum viscosum* (type XII), and *Pseudomonas* sp. (type XII) were purchased from Sigma Chemical Co. Superdex 75 and Sepharose 4B were products of Pharmacia Co. All other reagents were purchased in the purest form available from commercial sources.

Assay of Lipase and Its Inhibitory Activity. The procedures were adapted from that previously described by Satouchi and Matsushita (1976). To determine the lipase activity, soybean oil emulsion was prepared by vigorous stirring with 10 mL of 5% gum arabic and 1 mL of soybean oil and used as a substrate in the experiment. The assay was performed on the incubation for 5 min at 37 °C in a 3 mL centrifuge tube in a total volume of 200 μL of 125 mM Tris-HCl buffer, pH 7.4, containing 1.25 mM calcium acetate, and 100 μL of lipase (500 units/mL), followed by further incubation for 10 min after the addition of 100 μL of soybean oil emulsion. The hydrolysis of soybean oil was assayed by measuring free fatty acids liberated in the reaction mixture according to the method of Duncombe (1963) using linoleic acid as the standard. To determine the lipase 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 determined. The inhibitory activity of the protein was expressed by absorbance at 440 nm on the amounts of released fatty acids, or as inhibitor unit. One inhibitor unit was arbitrarily defined as the amount of protein that decreases lipase activity to 50% of the initial value.

Preparation Steps of LI. All purification procedures were carried out at about 4 °C, and centrifugations were performed at 10000*g* for 30 min in a refrigerated centrifuge.

Step 1. Wheat flour [1 kg, 8% (w/w) protein on a moisture-free basis] of commercial origin was extracted with 3 L of 60% ethanol to eliminate endogenous lipids to which proteins might

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be adsorbed. The mixture was centrifuged and the precipitate homogenized in 3 L of 0.1 M Tris-HCl buffer, pH 7.4, containing 1 mM calcium acetate. The homogenate was filtered through four sheets of gauze. The filtrate was centrifuged to be clear. To the resulting supernatant was added solid ammonium sulfate to give 10% saturation, and then the mixture was allowed to stand for 1 h. The precipitate was removed by centrifugation. The resulting solution was concentrated by ultrafiltration with an ultrafiltration module (nomimal molecular weight cutoff, 100 000, Asahi Chemical Industry Co., Ltd., Tokyo). The concentrates were lyophilized and named crude LI.

Step 2. The crude LI (5 g) was dissolved in 100 mL of 2 M NaCl solution, and the resulting supernatant was chromatographed on a butyl Toyopearl 650C column (6 mL, Toso Co. Ltd., Tokyo). The inhibitory activity of each fraction was assayed. The fractions with the inhibitory activity were pooled and then concentrated under a vacuum.

Step 3. The concentrate was placed on the top of a Superdex 75 column (2 × 95 cm) previously equilibrated with 0.1 M Tris-HCl, pH 7.4, containing 6 M urea. Elution was carried out with the same buffer at a flow rate of 1 mL/min.

Step 4. Porcine pancreatic lipase (10 mg) was immobilized to 4 g of CNBr-activated Sepharose 4B (Tan-Wilson *et al.*, 1976). The lipase–Sepharose column (4 mL) was washed with 10 mM Tris-HCl buffer, pH 7.2, containing 0.15 M NaCl and charged with the dialyzed solution in step 3. After all unbound protein had been washed out with the charging buffer, the protein was eluted from the column with linear gradient of NaCl from 0.15 to 1.0 M in the same buffer.

Polyacrylamide Gel Electrophoresis. SDS–PAGE was performed according to the method of Laemmli (1970). Protein was stained with a solution of 0.2% Coomassie brilliant blue R-250 in water/2-propanol/acetic acid (5:5:1, by vol). Two-dimensional electrophoresis was carried out using an Investigator 2-D electrophoresis system (Millipore, Bedford, MA). For Western blotting, proteins on an SDS–PAGE plate were transferred electrically to Immobilon P membrane (Millipore), using a Milliblot-Graphite Electroblotter II system (Millipore) at 100 V for 30 min.

Analytical Methods. The protein concentration was routinely determined by absorbance at 280 nm. Protein content of the various solutions was estimated by the colorimetric method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Amino Acid Sequence Determination. The amino acid sequence in the amino-terminal portion of the purified protein was determined using a protein sequencer (Model LF-3000, Beckman; Matsudaira, 1987).

Determinations of Heat and pH Stability. The heat stability was examined at pH 7.2. Each sample was heated at the desired temperature (65 and 80 °C) for 1 h, and after cooling, the residual activity was determined at 37 °C. The effect of pH was examined by the determination of remaining inhibitory activity after incubation at 37 °C for 60 min at various pH values.

RESULTS

Purification of LI. Hydrophobic column chromatographic studies of crude LI preparation, as described in step 2, revealed that the proteins with lipase inhibitory activity were effectively eluted in adsorbed fractions (Figure 1). The unadsorbed materials having no inhibitory activity were washed out from the column with the buffer containing NaCl as shown in Figure 1. The inhibitors were eluted from the column with the buffer not containing NaCl (fractions 92–108) and linear gradient of ethanol from 0 to 99.5% (fractions 136–180). The main peaks of inhibitory activity appeared in the ethanol fraction. The ethanol fraction (LI-E) was pooled and concentrated to remove ethanol under a vacuum. LI-E was applied to a Superdex 75 column according to the procedure of step 3. As shown in Figure 2, one peak (fractions 68–80, LI-Ea) of the inhibitory activity in the four peaks was pooled and dialyzed against 10 mM Tris-HCl buffer, pH 7.2, containing 0.15 M NaCl. The dialysate of LI-Ea was applied to the lipase–Sepharose 4B affinity column chromatography as described in step 4. After unadsorbed material was washed out through the column, a single peak with inhibitory activity was detected by OD at 280 nm (Figure 3). The peak was collected and then concentrated (LI-Ea-1).

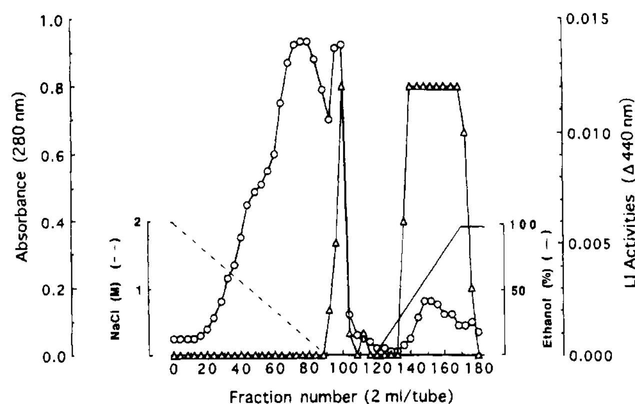

Figure 1. Hydrophobic chromatography of crude LI preparation. Butyl Toyopearl 650C was equilibrated with 0.1 M Tris-HCl buffer, pH 7.4, containing 2 M NaCl. The column was washed with the same buffer, and adsorbed proteins were eluted with a linear gradient from 2 to 0 M of NaCl, followed by elution with the above-mentioned buffer without NaCl and then with a linear ethanol gradient from 0 to 99.5% (v/v). (○) Absorbance at 280 nm; (△) LI activity at 440 nm.

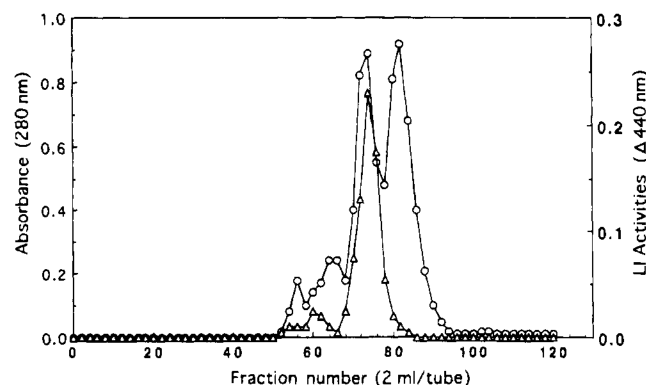

Figure 2. Gel filtration of LI-E on a Superdex 75 column. LI-E dialyzed against 0.1 M Tris-HCl buffer, pH 7.4, containing 6 M urea was applied on the column (2 × 95 cm) previously equilibrated with the same buffer, containing 6 M urea. The elution rate was 1 mL/min, and 2 mL fractions were collected. (○) Absorbance at 280 nm; (△) LI activity at 440 nm.

SDS–PAGE of LI-Ea-1 revealed two peaks with molecular mass of approximately 28 and 25 kDa, indicating that these proteins were the substances responsible for the activity. The appearance of only two bands showed a significant degree of purification in the above procedures up to this step. The specific activity of the purified protein was 90510 units/mg of protein, and purification factor was 285.5-fold, as summarized in Table 1.

Properties of LI-Ea-1. LI-Ea-1 was analyzed by two-dimensional electrophoresis. Three major spots (LI-Ea-1-1, LI-Ea-1-2, and LI-Ea-1-3) and minor spots were detected. After electrophoretic transfer to the membrane, each protein of the three major spots was extracted from the membrane. Each extracted protein was confirmed to be pure by two-dimensional electrophoresis, and the amino acid sequences in the N-

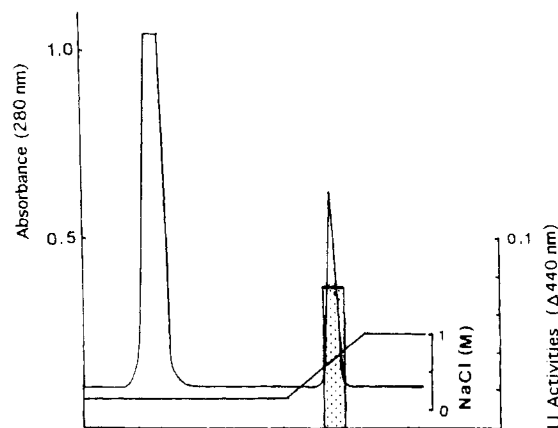

Figure 3. Lipase−Sepharose 4B affinity chromatography of LI-Ea. The lipase−Sepharose column was equilibrated with 10 mM Tris-HCl buffer, pH 7.2, containing 0.15 M NaCl, and LI-Ea was subjected to the lipase−Sepharose 4B column. After unadsorbed protein was washed out through the same buffer, a single peak with inhibitory activity was eluted with a linear gradient of NaCl from 0.15 to 1.0 M in the same buffer. (Dotted area) LI activity at 440 nm.

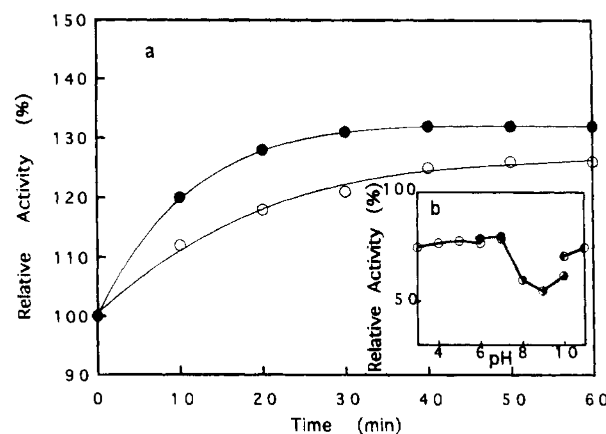

Figure 4. Effect of temperature (a) and pH (b) on stability of LI-E. (a) LI-E was dissolved in 125 mM Tris-HCl buffer, pH 7.2, and left to stand at 65 (●) and 80 °C (○) for 1 h. The remaining inhibitory activities were measured. (b) LI-E was dissolved in each buffer as described below and incubated at 37 °C for 1 h. The remaining inhibitory activities were measured. (○) 0.1 M potassium acetate buffer; (●) 0.1 M potassium phosphate buffer; (◑) 0.1 M Tris-HCl buffer; (◐) 0.1 M potassium carbonate buffer.

Table 1. Purification of Lipase Inhibitors from Wheat Flour

purification step	yield (mg)	specific activity (units/mg of protein)	purification factor
butyl Toyopearl	240.0	317	1
Superdex 75	20.2	13100	41.3
lipase−Sepharose 4B	5.9	90510	285.5

Table 2. Amino Acid Sequence from N-Terminal Amino Acid Residue

	residue no.								
isolate	1	2	3	4	5	6	7	8	9
LI-Ea-1-1	Arg	Ser	Ala	His	Glu	Pro	Gln	Gln	Pro
LI-Ea-1-2	Arg	Ser	Ala	His	Glu	Pro	Gln	Gln	Pro
LI-Ea-1-3	Arg	Ser	Ala	His	Glu	Glu	Gln	Gln	His

terminal regions from the N terminus to the ninth residue of these proteins were aligned and compared (Table 2). A high degree of homology in the amino acid sequence was observed between LI-Ea-1-1 and LI-Ea-1-2 (molecular masses of 25 kDa), and two residues of LI-Ea-1-3 (molecular mass of 28 kDa) were different from the other isolates. Thus, a high degree of homology among the three isolates could be demonstrated. They were suggested to be genetic variants. As described above, the proteinous inhibitor of lipase from wheat flour was purified to homogeneity by ultrafiltration, hydrophobic column chromatography, gel filtration, affinity chromatography, SDS−PAGE, and two-dimensional electrophoresis.

Thermal and pH stabilities of LI were examined, and the results are shown in Figure 4. LI-E was stable against high temperatures of 65 and 80 °C and apparently activated by heat treatment (Figure 4a). However, the inhibitory activity of LI-E heated at 65 °C was greater than that at 80 °C. And LI-E was stable in the pH range 3−7, indicating that the remaining inhibitory activity was minimum at pH 9 (Figure 4b).

Effect on Various Lipases. Lipase from porcine pancreas was fully inhibited by the partially purified proteins of LI-E (inhibitory activity units/mg of protein: 12930), while the activity of the enzyme from *C. cylindracea* was partially inhibited (inhibitory activity units/mg of protein: 6120); the activities of the enzymes from the other microbacteria described under Materials and Methods were not inhibited.

DISCUSSION

The proteinous inhibitors of lipase from wheat flour in the present stady were shown to have a unique characterization having the potent activity caused by direct interaction between lipase and inhibitor. This was demonstrated by the results of chromatography on an affinity column of lipase−Sepharose to which lipase inhibitors were bound and by the inhibitory mechanism on their direct interaction as suggested in the preincubation treatments. This is not consistent with previous observations of soybean and wheat proteins that are known to affect lipolysis, presumably through their binding to the oil−water interphase (Satouchi *et al.*, 1974; Satouchi and Matsushita, 1976).

Borgstrom and Erlanson (1978) and Blackberg *et al.* (1979) showed that pancreatic lipase activity was inhibited by hydrophobic proteins such as serum albumin or β-lactoglobulin in the absence of colipase and bile salts. Gargouri *et al.* (1984) isolated a protein that inhibited pancreatic lipase activity from soybean seeds and showed that inhibitory properties of the purified protein were very similar to those of serum albumin or β-lactoglobulin. The mechanisms underlying the observed effects are still poorly understood, but no interaction between pancreatic lipase and soybean inhibitory protein could be observed. Therefore, the inhibitory mechanisms apparently were not the same as the one based on the soluble proteins from wheat flour as described in this study.

Lipases from porcine pancreas and *C. cylindracea* were fully and partially inhibited, while inhibitory activity against lipases from some microbacteria remained unchanged under the same experimental conditions. This finding might reflect differences in the affinities of these lipases to LI-E. Furthermore, lipase inhibitory activity was increased by heat treatments and more effective when LI-E was heated at 65 °C than at 80 °C. This probably shows that the structure of the inhibitory protein was partially denatured and interaction between the protein and lipase readily occurs.

Pancreatic lipase is the key enzyme of dietary triacylglycerol digestion and assimilation. It acts at the surface of emulsified lipid droplets, and this interfacial activation distinguishes lipases as a subclass of es-

terases (Verger, 1984). Even though porcine pancreatic lipase is the best studied triacylglycerol hydrolase, the lipolytic mechanism of this enzyme is still poorly understood. These results can be interpreted to indicate that pancreatic lipase is a serine-type esterase with Ser152 as the active site serine (Chapus *et al.*, 1988; Guidoni *et al.*, 1981). Inhibitors are bound as an ester to serine 152 of the lipase. The detailed inhibitory mechanism must also be demonstrated in the sample of protein described here.

It is noteworthy that inhibitory proteins were isolated from wheat flour but the physiological significance of the proteins may be questioned. The proteins, as isolated from wheat flour in the present study, may well play a role during fat digestion and assimilation *in vivo*. Further studies must be undertaken to compare the respective effects of inhibitory protein concentrates and raw wheat flour on lipid digestion and metabolism. Since plant proteins will constitute a large part of dietary proteins in the future, the investigation of enzymic effectors of plant origin is of prime importance.

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