# Pancreatic Lipase Effectors Extracted from Soybean Meal

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The activity of porcine pancreatic lipase is shown to be affected by proteinaceous compounds extracted from raw soybean meal. Both activators and inhibitors were detected. They are heat stable and only moderately susceptible to proteolysis. Their apparent effects on lipolysis are assumed to be due to interaction with the substrate emulsion.

The nutritional merits of certain plants as a source of protein are often outweighed by their capacity to synthesize compounds (very often proteins affecting enzymic activities) unsuitable for human or animal consumption. These naturally occurring toxicants induce adverse physiological effects (for a review, see Liener, 1969, 1976). Since plant proteins will constitute a larger part of dietary proteins in the future, the investigation of enzymic effectors of plant origin is of prime importance.

Two recent publications have described the purification of a heat-labile proteinaceous inhibitor of pancreatic lipase from undefatted soybean meal (Mori et al., 1973; Satouchi and Matsushita, 1976). This prompted us to determine whether heat-treated soymeal extracts could similarly affect pancreatic lipase activity. This proved to be the case, but due to significant polydispersity a proper purification of the heat-stable lipase effectors was not achieved. However, the results presented here indicate that a purified extract representing 7% of the total soymeal protein contains efficient activators and inhibitors of pancreatic lipase.

## MATERIALS AND METHODS

**Chemicals.** Gum arabic was purchased from Siegfried, cold-pressed olive oil from Nuxo, and lyophilized porcine pancreatic lipase from Fluka. All other reagents were purchased in the purest form available from commercial sources.

**Protein and Sugar Determinations.** The protein content of soybean meal was determined with an Automatic Nitrogen Analyzer (C. Erba Co.), using the legally recognized conversion factor of 6.25 (American Soybean Association and Association of Official Analytical Chemists). The protein content of the various solutions was estimated by the Folin method (Lowry et al., 1951), using BSA (bovine serum albumin) as a standard. Total sugars were evaluated by the phenol–sulfuric acid method (Dubois et al., 1956).

**Soy Extract Purification Steps.** (1) Nondefatted raw soybean meal (39% w/w protein, on a moisture free basis) was stirred for 90 min at room temperature with 0.1 M Tris–HCl buffer, pH 7.4 (meal/buffer weight ratio, 1:10), and centrifuged for 30 min at 30 000*g*. The pellet was discarded, and the resulting supernatant contained ca. 75% of the total protein. (2) The supernatant was heated at 100 °C for 30 min and centrifuged for 45 min at 50 000*g*; this step denatured and removed three-fifths of the extracted proteins. (3) The proteinaceous precipitate obtained by selective ammonium sulfate precipitation (between 25 and 50% saturation) was dialyzed against 0.1 M Tris–HCl buffer (pH 7.4). This did not result in a complete redissolution, and the fine residual suspension was disposed of by a 30-min centrifugation at 100 000*g*. The supernatant contained 10% of the extracted proteins (7.5% of the total protein) and 2% of the extracted sugars. (4) The purified extract was processed in the concentration/dialysis mode against distilled $H_2O$ in an Amicon Diaflo Cell (PM 30 filter). The protein concentration in the final extract was adjusted to 45 mg/mL, and the protein content was 7% of the total soybean meal protein. This extract must be free of any buffer, because of its subsequent use in pH-stat assays (see below).

**Lipase Assay.** Gum arabic (2 g) was dissolved in hot distilled $H_2O$ (180 mL). After cooling to 4 °C, olive oil was added (20 mL), and the mixture was emulsified in a Sorvall homogenizer for 10 min. The resulting stock emulsion was stable for several hours if kept at 4 °C. The standard substrate emulsion was made up of 10 mL of stock emulsion, 20 mL of 4.5 mM $CaCl_2$, and 0.3 mL of 20% sodium cholate (pH adjusted to 8.5 with NaOH, room temperature; final sodium concentration = 6 mequiv/L). Addition of 1 mL of lipase solution (0.05% in 3 mM $CaCl_2$, adjusted to pH 8.5) initiated the enzymic reaction. Sodium and calcium ions, as well as bile salts, are required to ensure a proper lipolysis rate (Desnuelle, 1972). Lipolytic activity was monitored by recording the consumption of 0.05 N NaOH used to stabilize the pH at 8.5 (Metrohm pH-stat). The progress curves (NaOH consumption vs. time) were straight lines for about 2 min, which allowed for an accurate determination of the initial rates. The activity of lipase was found to be 5.2 $\mu$equiv fatty acids liberated $min^{-1}$ $mg^{-1}$ enzyme (fresh solution). The purified soy extract was used in the experimental set up (addition to the lipase solution or to the substrate emulsion) as described in the Results section.

## RESULTS

**Stability of the Lipase Solutions.** The lipase solutions were always prepared and kept at room temperature, in order to ensure complete solubility of the lyophilized enzyme. A significant decrease of lipase activity was noted on standing (Figure 1, curve A, initial rates). However, the denaturation process appeared to be partially reversible, since the decrease of initial rates was paralleled by the appearance of an upward curvature for the progress curves. Such a curvature is evidenced by the fact that curve B on Figure 1 (rates 5 min after initiation of the reactions) has always higher ordinates than curve A. Effective protection against denaturation was afforded by BSA, since lipase solutions prepared in 1% BSA (with 20 mg of BSA/mg of lipase) were found to be stable for at least 4 h (results not shown), with an activity representing 100–115% of the standard activity. This slight activation effect will be discussed below. HSA (human serum albumin) exerted a similar action.

**Activation of Lipase Activity by the Soy Extract.** No endogenous lipase activity could be detected in the purified soy extract (because of the heat treatment included in the purification steps). Curve C on Figure 1

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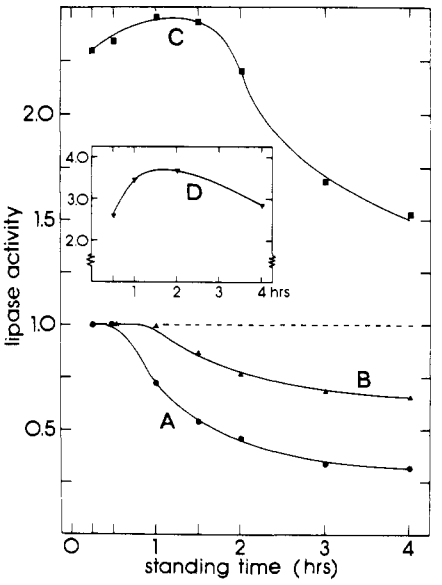


**Figure 1.** Effect of preincubation of lipase with the soy extract: abscissa, zero time corresponds to the preparation of the lipase solution; ordinate, lipase activity expressed in arbitrary units (the standard initial rate at zero time was arbitrarily set at 1.0); curve A, initial rates for the standard assay; curve B, rates for the standard assay, 5 min after initiation of the reactions; curve C, initial rates in the presence of 13 mg of soy extract/mg of lipase; curve D, initial rates in the presence of 45 mg of soy extract/mg of lipase (insert).

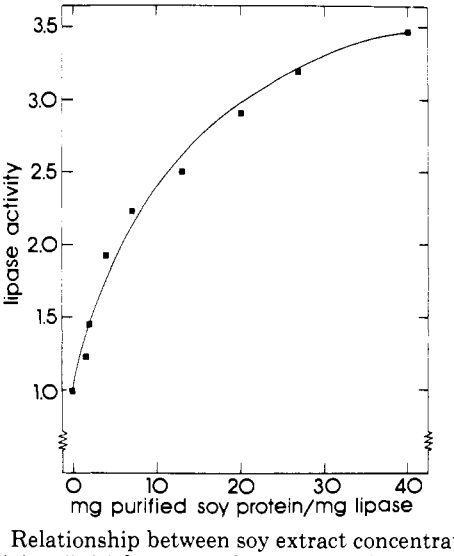


**Figure 2.** Relationship between soy extract concentration and lipase activity (initial rates, arbitrary units), after ca. 1 h preincubation of the enzyme with the soy extract.

shows therefore that preincubation of the pancreatic lipase with the extract promoted a significant activation of the enzyme, reaching a maximum after ca. 90 min. The subsequent falling off of curve C shows that the extract was unable to completely stabilize the enzyme, unlike BSA. However, the relative decrease of lipase activity is less noticeable for curve C than for curve A, which evidences a protective effect of the purified soy proteins for lipase. An increase of the soy extract concentration promoted an enhancement of the protective effect (curve D).

The relationship between the concentration of the purified soy proteins and lipase activity is given in Figure 2. The activation effect was significant, since a concentration of 40 mg of soy extract/mg of lipase (i.e., 20 mg of soy extract/30 mL of emulsion) promoted ca. 250% activation. The proteinaceous nature of the activator(s)

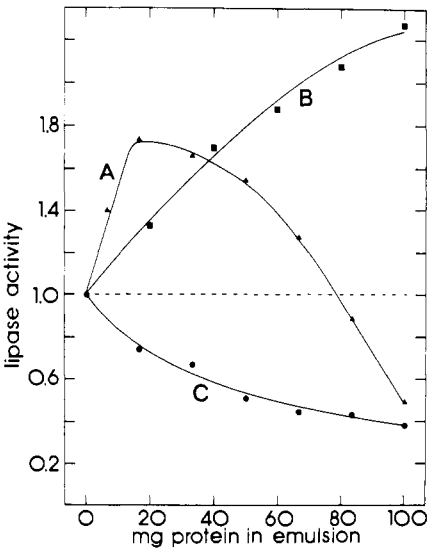


**Figure 3.** Relationship between purified soy extract or BSA concentration and lipase activity (initial rates, arbitrary units), when the proteins were added to the substrate emulsion prior to initiating the assays: curve A, fresh lipase (prepared ca. 20 min before use); soy extract added to the emulsion; curve B, fresh lipase; BSA added to the emulsion; curve C, aged lipase (prepared ca. 2 h before use); soy extract added to the emulsion. For each curve, the initial rate with a standard emulsion was arbitrarily set at 1.0.

was established by the fact that no activation could be detected if the extract had been previously digested by pronase (24 h at 37 °C, 0.5 mg of pronase/mg of soy extract).

**Inhibition of Lipase Activity by the Soy Extract.** When substrate emulsions containing the purified soy proteins in various concentrations were assayed with standard lipase, both activation and inhibition were detected, depending on the lipase solution used (see Figure 3). Curve A (fresh lipase) shows a significant activation effect up to 20 mg of soy extract/30 mL of emulsion. This concentration corresponds to 40 mg of soy extract/mg of lipase, where saturation becomes perceptible for the activation effect (see Figure 2). Curve A (Figure 3) indicates that an increase of the soy extract concentration did not promote higher activation. On the contrary, activation was gradually antagonized, and after addition of more than 80 mg of soy extract the net effect was a significant inhibition. This indicates therefore that the soy extract also contained pancreatic lipase inhibitors. The inhibitory effect disappeared on trypsin treatment, which established the proteinaceous nature of these inhibitors (see below).

Curve B (Figure 3, fresh lipase) shows that addition of BSA to substrate emulsions resulted in an increasing activation effect (the results were not significantly different when HSA and/or aged lipase were used). The addition of hemoglobin, however, had an inhibitory effect (ca. 50% inhibition for 100 mg of hemoglobin/30 mL of emulsion).

Curve C (Figure 3) indicates that inhibition was found when aged lipase was used with soy extract-containing emulsions. Comparison between curves A and C suggests therefore that ageing altered the response of lipase to its effectors found in the soy extract since aged lipase was susceptible to inhibition only. Figure 4 shows in more detail how the response of lipase to its soy extract effectors changed in function of time. Up to 90 min, the activation effect outweighed any inhibition, but thereafter the situation was reversed. The two curves are not significantly different, although curve B suggests that renatured lipase (see Figure 1 and connected comments) was less sensitive

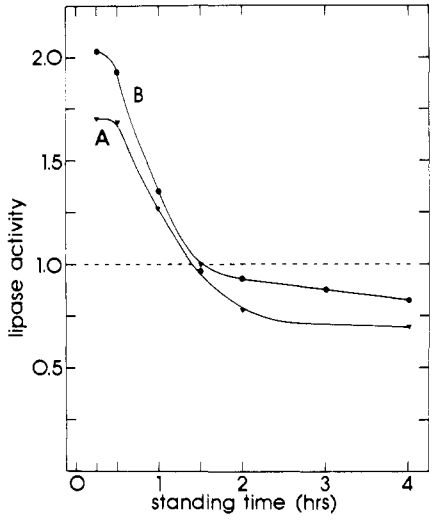


**Figure 4.** Evolution with time of the response of lipase to the purified soy proteins added to the substrate emulsion (23 mg/30 mL of emulsion): abscissa, zero time corresponds to the preparation of the lipase solutions; ordinate, for each experiment, the actual rate was compared to that with a standard emulsion; the values reported on the graph are the ratios between these rates; curve A, relative initial rates; curve B, relative rates 5 min after initiation of the reactions.

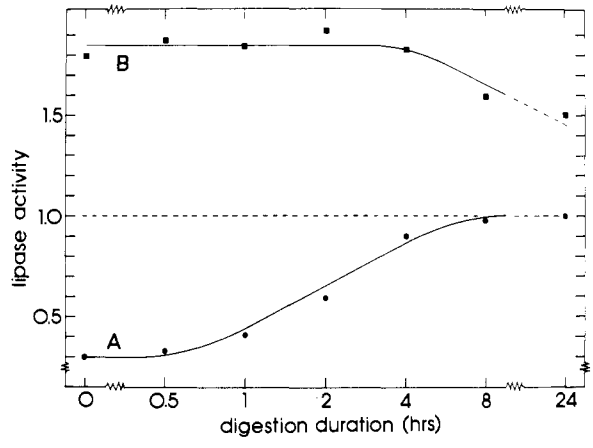


**Figure 5.** Effect of tryptic digestion on the soy extract proteins: abscissa, digestion duration (at 37 °C); ordinate, relative initial rates (lipase assays with the digested extracts); digestion mixture, 16 mL of 0.1 M Tris–HCl buffer (pH 7.4, with 3 mM $CaCl_2$), containing 180 mg of soy extract protein and 5 mg of trypsin; digestion termination, the above mixture was heated for 20 min at 100 °C, centrifuged to discard any precipitated protein, and dialyzed for 24 h against distilled $H_2O$; lipase assays, 10 mL or 4 mL of the above dialyzed solution were used in the preparation of the substrate emulsions or the lipase solutions, respectively; curve A, aged lipase, soy extract added to the substrate emulsions (to detect inhibitors); for each experiment, the actual initial rate was compared to the initial rate obtained with a standard emulsion; the values reported on the graph are the ratios between these rates; curve B, fresh lipase, preincubated for 1 h with the soy extract (to detect activators); for each experiment, the actual initial rate was compared to the initial rate obtained with a standard lipase; the values reported on the graph are the ratios between these rates.

to inhibition, and was therefore comparable to fresh lipase.

Figure 5 shows the distinct time courses of tryptic digestion for lipase activators and inhibitors. The two kinds of effectors were fairly resistant to proteolytic attack, but were destroyed on prolonged trypsin treatment. They were therefore of proteinaceous nature, but their different sensitivities to proteolysis indicate that they were separate entities.

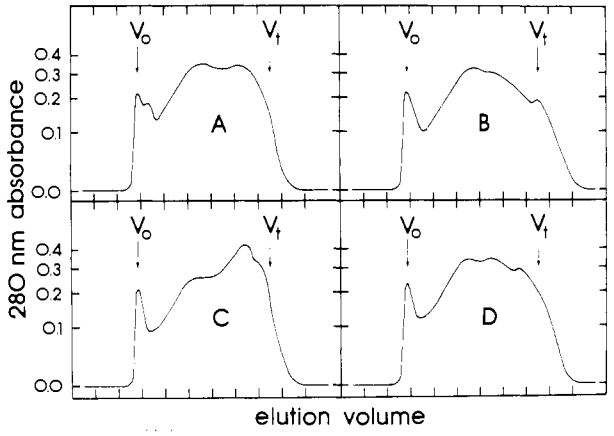


**Figure 6.** Elution profile of the purified soy extract on Sepharose 6B, in various buffers ($V_o$, void volume; $V_t$, total volume): curve A, in 0.1 M Tris–HCl buffer (pH 7.4); curve B, in 0.1 M sodium phosphate buffer (pH 7.4); an increase of the buffer concentration up to 0.25 M increased the height of the last peak on the right-hand part of the elution profile; curve C, in 0.25 M sodium phosphate buffer (pH 8.8); curve D, in 0.25 M sodium phosphate buffer (pH 6.0).

**Elution Profiles on Sephadex and Sepharose Gels.** Figure 6 shows that the mol wt (molecular weight) distribution of the extract corresponded to the fractionation range of a Sepharose 6B gel. However, curves A to D on Figure 6 indicate that an efficient separation was not achieved using different buffers, although four main components were revealed by curve A (seven components were resolved by gradient polyacrylamide gel electrophoresis, i.e., a method which separates proteins according to charge *and* molecular weight). The biological activity (lipase activation and inhibition) was similarly spread over the whole fractionation range. These results explain why gel chromatography could not be included in the purification steps. The elution peak moving with the void volume ($V_o$, Figure 6) represented very large proteins (mol wt $> 5 \times 10^6$), which were not affected by the varying buffer conditions (i.e., the same peak is seen unchanged on each curve and represents ca. 5% of the eluted material). The rest of the elution profile was, however, affected by varying conditions. Moderate changes, with no possible effect on covalent bonds, were therefore able to promote the disaggregation of high molecular weight material. This result indicates that the purified proteins were prone to random aggregation (this could be partially due to the heat treatment; Saio et al., 1968).

The purified extract behaved similarly when applied to a DEAE–Sephadex ion-exchange gel, since development of the column with a linear 0.0–0.5 M NaCl gradient did result in an arch-like elution profile. This is indicative of a fairly continuous charge distribution, which would be in agreement with the noncovalent random aggregation hypothesis. This might explain why stepwise precipitation of the extract between pH 4.0 and 5.8 was unsuccessful as a tentative purification step, i.e., both lipase activators and lipase inhibitors were detected in all supernatants and all precipitates. This was corroborated by the fact that both kinds of lipase effectors were found to be present in soy isolate as well as in soy whey. This in turn implies that the polydispersity of the purified proteins was not due to the heat treatment only, since such a treatment was not used in the preparation of soy whey and isolate.

**Partial Precipitation of the Purified Extract with Calcium Ions.** Maximum precipitation was obtained for the fairly low $CaCl_2$ concentration of ca. 20 mM, whereas a concentration of 120 mM was sufficient to ensure an

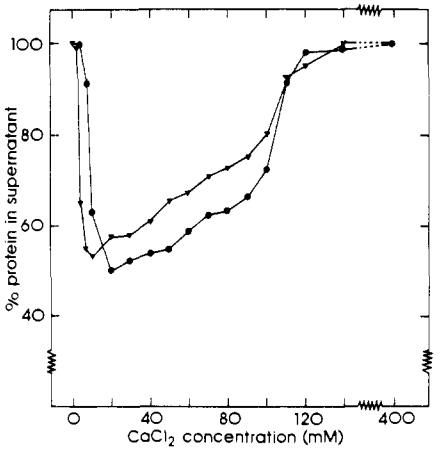


**Figure 7.** Precipitation pattern of the purified soy extract by $CaCl_2$: curve A (▼—▼), 100% on the ordinate = 8 mg of protein/mL (pH 6.5, without any buffer); curve B (●—●) 100% on the ordinate = 40 mg of protein/mL (pH 6.5, without any buffer).

almost complete redissolution. This is an unusual precipitation pattern (see Figure 7). $MgCl_2$, $SrCl_2$ as well as $BaCl_2$, were found to promote similar effects. These results complement those obtained in earlier studies, which suggested the possibility of soybean meal protein fractionation by selective extraction with salt solutions (Smith et al., 1938; Wolf and Briggs, 1956). It appears, however, that this approach has not been studied further (Wolf, 1972). When compared to curve B on Figure 7 (100% = 40 mg of protein/mL), curve A (100% = 8 mg of protein/mL is seen to be slightly shifted to the left. However, the shift is too small to account for any stoichiometric relationship between the protein and the precipitating agent. Moreover, stepwise precipitation with $CaCl_2$ was unable to bring about a proper separation of the pancreatic lipase effectors described in this report.

## DISCUSSION

Proteins are known to affect lipolysis, presumably through their binding to the oil–water interphase. Such a binding can increase or decrease the interfacial area accessible to lipase, as well as prevent the rapid unfolding of lipase due to the interfacial surface tension (Brockerhoff, 1971). The net effect can be an apparent activation of inhibition of the enzyme, although the primary action of the effectors is exerted on the substrate.

The effect of BSA on pancreatic and microbial lipases is well documented (Brockerhoff, 1971; Sémériva et al., 1969). This report shows that heat-stable proteins from soybean meal are even more active effectors of lipolysis. Inhibition or activation is revealed depending on the experimental conditions (e.g., preincubation with the enzyme or with the substrate), but a definite mechanism of action cannot be deduced from the present results. The response of the enzyme changes when the native structure is gradually altered on standing. This indicates that the effectors may interfere with the conformational alteration occurring when lipase is adsorbed on the hydrophobic substrate interface.

The failure to achieve a proper purification suggests that the observed effects must be exerted by classes of compounds rather than by sharply defined entities. In this connection, it is worth pointing out that the purification procedure of the heat-labile lipase inhibitor described by Satouchi and Matsushita (1976) was also indicative of significant polydispersity (the inhibitor described by these authors was only a small proportion of the active material as detected on Sephadex and ion-exchange columns). Also, it has to be kept in mind that the well-documented soybean anti-tryptic activity is similarly due to several compounds (for a review, see Liener, 1969).

The conformational stability of the effectors described in this report implies that after ingestion they may remain active in the digestive tract and thereby affect intestinal lipolysis. The net effect is, however, difficult to predict, as the distinction between fresh and aged lipase hardly applies to in vivo conditions. Moreover, the presence of other proteins included in the diet may interfere under such conditions, as indicated by the results pertaining to BSA, HSA, and hemoglobin.

## NOTE ADDED IN PROOF

After this manuscript was completed, additional experiments were performed using triacetin as the substrate. In this case, the purified lipase effectors were not detected to any significant extent. This substantiates the assumption that the apparent activation or inhibition of lipolysis reported in this communication were mainly due to interactions between the proteinaceous effectors and the substrate (i.e., olive oil).

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