Purification of pancreatic lipase via its affinity for bile salts and apolar surfaces

William E. Momsen and Howard L. Brockman

The Hormel Institute, University of Minnesota, Austin MN 55912

Abstract An improved method for the purification of pancreatic lipase in milligram amounts is described. Only one batch step and one chromatographic step are required to produce a lipase–colipase complex and a lipase largely free of colipase. Both species are lipid free; physical and kinetic data indicate that the predominant lipase species is lipase B.

Supplementary key words colipase · taurodeoxycholate · taurocholate · protein adsorption · siliconized glass beads

Pancreatic lipase has been the subject of extensive study, particularly since lipolysis serves as a representative example of enzymatic reactions that occur at phase boundaries (1, 2). Difficulties inherent in the purification of this enzyme have been the removal of a tightly bound acidic phospholipid (3) and a protein cofactor, colipase (4, 5). Phospholipid was removed by the incorporation of a butanol flotation step between the standard chloroform–butanol extraction step and column chromatography (3). Overall, the purification of lipid-free pancreatic lipase which contains some colipase requires a total of two batch steps and three chromatographic steps after making the initial solvent extracted powder of porcine pancreas (3). The removal of colipase, which varies from 0 to 0.3 mol per mol of lipase, has recently been accomplished by treating the enzyme with guanidine hydrochloride under reducing conditions followed by chromatography on Sephadex G-100 (4). Another procedure for lipase purification, based on adsorption to concanavalin A, has been published (6) but it requires four chromatographic steps. Lipid and colipase analyses of the product were not reported.

Our investigations of the role of colipase and bile salts in lipolysis showed that lipase is readily adsorbed to apolar surfaces (7) and that this adsorption can be prevented by sodium taurodeoxycholate at concentrations near its critical micelle concentration (8). These properties of the purified enzyme suggested that substantial purification of lipase from crude extracts could be achieved through adsorption of the enzyme to an apolar surface followed by desorption with TDC. With such a procedure, we obtained a 10-fold purification of the enzyme, and subsequent chromatography of the resulting material on DEAE-cellulose yielded lipid-free lipase, a part of which was largely free of colipase as well. The procedure requires much less time than existing methods and can easily be scaled to yield between 1 and 100 mg of lipase. The details of this procedure and the characterization of the lipase obtained are described below.

MATERIALS AND METHODS

Materials

The purifications of lipase B, lipase A, and colipase used in the assays described below were described previously (8). The method used was essentially that of Verger et al. (3) except that a second DEAE-cellulose column was used instead of a CM-cellulose column (9). This particular preparation of lipase B contained less than 1% colipase when assayed as described below.

The preparation of substrate solutions containing tributyrin or tripropionin has been described elsewhere (8). TDC (>96% pure), TC (>98% pure), and DFP were obtained from Calbiochem, San Diego, CA. DFP was stored as a 1 M solution in isopropyl alcohol. Microgranular DEAE-cellulose (DE-32) was purchased from Whatman, Clifton, NJ, and reagents for polyacrylamide gel electrophoresis were from Bio-Rad Laboratories, Richmond, CA. All other chemicals were reagent grade.

Glass micro beads (Class III, size MS-XL, −270 + 1000 mesh) used in the purification were obtained from Cataphote Division, Ferro Corporation, Jackson, MS. A waterproof coating was chemically bonded to the bead surface during manufacture. For the kinetic

Abbreviations: TDC, sodium taurodeoxycholate; TC, sodium taurocholate; DFP, diisopropylfluorophosphate.

experiments described below, siliconized glass beads were prepared as previously described (8).

Protein

The concentration of protein was estimated spectrophotometrically from absorbances at 280 and 260 nm using a nomograph based on protein and nucleic acid given by Warburg and Christian (10). Protein concentration of pure lipase or colipase samples was determined by absorbance using $E^{1\%}_{280} = 13.3$ and a mol wt of 50,000 for lipase (11) and $E^{1\%}_{280} = 4.0$ and mol wt 10,000 for colipase (12).

Measurement of lipase activity

During purification, lipase activity was determined by monitoring tributyrin hydrolysis with a pH-stat (Radiometer). The method was essentially that of Erlanson and Borgström (12) except that excess colipase and bile salt were used in order to minimize effects due to variable amounts of colipase at different stages of purification (13). To 2.0 ml of tributyrin emulsion (0.124 M tributyrin in 2 mM Tris, pH 6.5, 1 mM $CaCl_2$, 0.15 M NaCl) was added 11 pmol of colipase and TDC to give a final concentration of 4 mM. After stirring the solution at 1000 rpm for 1–2 min at 25°C, the pH was readjusted to 6.5, an aliquot containing 0–1 pmol of lipase was added, and the initial velocity of proton release was recorded. The titrant used was 5 mM NaOH. A unit of activity is 1 μmol of protons released per min.

For kinetic characterization of the purified enzyme, the tripropionin–siliconized glass bead assay system described by Momsen and Brockman (8) was employed.

Measurement of colipase activity

Colipase activity was measured as the ability to relieve bile salt-induced inhibition of lipase (12). Two assay systems were employed, depending on the relative amounts of lipase and colipase in the sample being tested. For samples containing more than 5% colipase on a mole basis, the assay used was essentially the same as the tributyrin assay for lipase described above, except that an excess of lipase B (11 pmol) was added instead of an excess of colipase. For those samples containing less than 5% colipase, sufficient lipase was present to complex all the colipase without additional, exogenous lipase B.

Fatty acid analysis

Lyophilized samples of lipase (about 1 mg) were dissolved in 1 ml of deionized water and extracted by the method of Bligh and Dyer (14). After evaporation of the $CHCl_3$, 2.0 ml of 1.57 N HCl in anhydrous methanol was added and the samples were heated for 1 hr at 90°C. The methyl esters obtained were dried, dissolved in redistilled Skelly B (bp 67–68°C), and analyzed by gas-liquid chromatography. A Packard 427 gas chromatograph fitted with a flame ionization detector and an aluminum column, 1/8″ OD by 6′, packed with 10% Silar 10C on 100/120 Gas Chrom Q (Applied Science), was used.

Phosphate analysis

Lyophilized samples of lipase (between 0.1 and 2 mg) were analyzed spectrophotometrically for total phosphate after perchlorate digestion according to Bartlett (15), except that the assay was scaled down from 10 ml to 1 ml total volume. Lipid-soluble phosphorus in an acidified sample was measured in the residue from a chloroform–methanol extract prepared by the procedure of Bligh and Dyer (14).

Bile salt analysis

A lyophilized sample (5.15 mg) of purified lipase was suspended in chloroform–methanol 2:1 (v/v) and the solvent fraction obtained after centrifugation was evaporated to dryness and assayed for taurodeoxycholate by the method of Eneroth and Sjövall (16). Sulfuric acid (1.1 ml, 65%) was added and the mixture was heated at 50°C for 15 min. After cooling, the absorbance was measured at 305 nm and the amount of taurodeoxycholate was calculated by reference to a standard curve.

Polyacrylamide disc gel electrophoresis

Polyacrylamide gels (7.5%) were run at pH 8.9 according to Maurer (17). Spacer gels were not used. The gel dimensions were 5 mm diameter × 6.5 cm and the protein bands were stained with Amido Black.

Ultrafiltration

Solutions were concentrated using an H1P10 hollow fiber cartridge (mol wt cut-off of 10,000) and DH4 cartridge adapter (Amicon Corp., Lexington, MA) at a flow rate of 2 liter/hr.

RESULTS

Purification procedure

Fresh porcine pancreas was obtained from a slaughterhouse and frozen immediately on solid CO_2. The tissue was then cut into 1-in cubes, trimmed of excess fat, and stored at −20°C. A delipidated powder was obtained from the defatted tissue by extraction with chloroform–butanol, acetone, and ether accord-

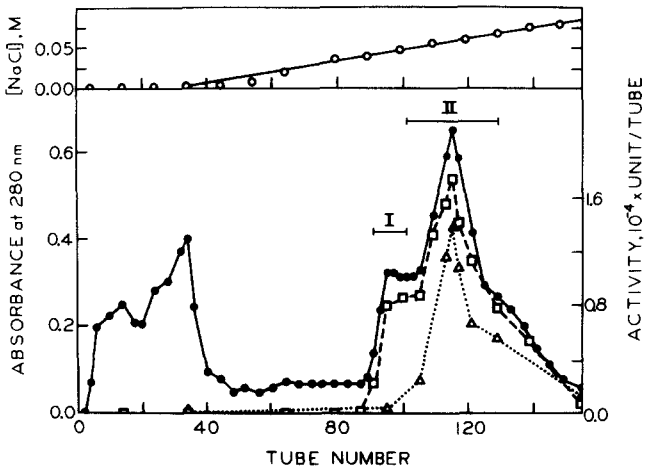

Fig. 1. Elution profile from DEAE-cellulose chromatography of the fraction 2 concentrate corresponding to the data in Table 1. Column dimensions, 1.0 × 21 cm; 350-ml linear gradient, 0–0.1 M NaCl in 5 mM Tris, pH 8, 3.3 mM $CaCl_2$; flow rate, 24 ml/hr; fraction volume, 3.0 ml; 4°C. ● —— ●, Absorbance at 280 nm; □ – – – □, lipase activity against tributyrin; △ ··· △, colipase activity; ○ —— ○, NaCl concentration.

ing to the method of Verger et al. (3). This powder was also stored at −20°C.

Protein was solubilized by addition of 20 g of delipidated powder to 200 ml of cold 1 mM K_2HPO_4, pH 7.5, 0.1 M NaCl, 1 mM DFP. After the pH was adjusted to 8.5–9.0, the solution was stirred for 1 hr at 4°C and centrifuged for 15 min at 27,000 *g*. The supernatant was adjusted to pH 6.5, recentrifuged as above, and the precipitate was discarded.

Glass beads (4 kg) were stirred in 3.0 liter of 10 mM K_2HPO_4, pH 6.5, 0.05 mM TDC (buffer A) intermittently for 0.5–1 hr at room temperature. The beads were then transferred to a 30.5-cm in diameter, table-top Buchner funnel fitted with rapid flow filter paper and washed with 3.0 liter of buffer A followed by 6.0 liter of 10 mM K_2HPO_4, pH 6.5, 1 mM EDTA, 0.2 mM TDC, 10 mM mercaptoethanol (buffer B) at room temperature. The bed height was 4 cm and the flow rate through the beads was about 200 ml/min.

The pancreatic extract was added to 8.0 liter of buffer B, yielding a protein concentration of about 1.5 mg/ml. The diluted extract was filtered through the beads at room temperature, followed by 10.0 liter of buffer B to yield fraction 1, which was discarded. Adsorbed lipase was then eluted from the beads with 3.0 liter of 1 mM K_2HPO_4, pH 7.5, 0.1 M NaCl, 1 mM EDTA, 3.5 mM TDC, 10 mM mercaptoethanol (buffer C) to yield fraction 2. The EDTA and mercaptoethanol were not required, but did help to prevent the formation of a precipitate during ultrafiltration.

Fraction 2 was concentrated at 4°C by ultrafiltration to a volume of 80 ml and the solution was made 0.5 mM in DFP to block any residual proteolytic activity. After a 10–15 min incubation, the concentrate was diluted 10- to 20-fold with 5 mM Tris, pH 8, and reconcentrated to remove the bile salt and exchange the buffer. This dilution and reconcentration was repeated twice more, with the final dilution–reconcentration being made in Tris buffer which also contained 3.3 mM $CaCl_2$.

The concentrate was applied to a 1.0 × 21 cm column of DEAE-cellulose equilibrated at 4°C with 5 mM Tris, pH 8, 3.3 mM $CaCl_2$. The column was washed with 10 ml of buffer followed by a 350-ml linear gradient from 0 to 0.1 M NaCl in the same buffer. The flow rate was 24 ml/hr and the fraction volume was 3.0 ml. The elution profile is shown in **Fig. 1.** A small fraction (5%) of the lipase activity applied was eluted in a subsequent 1.0 M NaCl wash of the column (not shown), but it had a low specific activity and was discarded. For each of the two peaks (I and II) containing lipase activity, fractions were combined and either stored at −20°C or dialyzed against water and then lyophilized. The results of this purification are given in **Table 1.**

The lipase eluting at 0.045 M NaCl was homogeneous as judged by polyacrylamide gel electrophoresis. It migrated with the same mobility on gels as lipase B purified as described under Materials and Methods. The second peak, eluting at 0.057 M NaCl, contained three bands. The upper band appeared identical to that from the first peak, as shown in **Fig. 2,** whereas the other two bands migrated with mobilities identical to those of lipase A and colipase purified as described under Materials and Methods. The colipase band in gels *b* and *c* was initially faint and faded during storage. Its position is indicated by the arrow in Fig. 2.

Comments on the purification

1) The beads are normally used as purchased and cleaning them more extensively than described above does not significantly improve the purification. The

TABLE 1. Purification of lipase from porcine pancreas

Sample	Protein	10^{-5} × Units	Yield	Specific Activity
	mg	*μmol/min*	*%*	*μmol/min/mg*
Extract	13600	10.0	100	73.5
Fraction 1	10700	4.53	45.3	42.3
Fraction 2	712	5.40	54.0	758
Concentrate of fraction 2	180	2.90	29.0	1610
DEAE peak I	8.3	0.38	3.8	4520
DEAE peak II	30.6	1.46	14.6	4780

beads are not reused because they are available in quantity and are inexpensive.

2) For a series of 11 adsorption experiments the percentage of lipase activity that adsorbed to the bead surface was 56 ± 11 (SD). Pilot studies have indicated that increasing the concentration of the diluted extract that is applied to the beads to above 2 mg per ml tends to decrease the percentage of lipase absorbed and that reducing the temperature to 4°C has no effect.

Up to 100% adsorption could be achieved by decreasing the total protein applied to the beads, but total adsorption/gram of beads was less and recovery was relatively poor. The conditions employed were those which give the best total recovery of lipase units/gram of beads considering that the starting material is easily prepared from porcine pancreas.

3) This preparation can readily be scaled up or down, provided the bed height for the beads is maintained at approximately 4 cm and the flow rate is maintained at 0.3–0.5 ml/min per cm² of funnel surface. Under these conditions, all solution volumes are kept proportional to the volume or weight of beads employed. Alternatively, the concentrates from two or more bead fractionation steps may be combined and chromatographed on a proportionately larger DEAE-cellulose column.

4) The 40–50% loss of activity which was routinely observed during the concentration and reequilibration of fraction 2 (Table 1) was confined to the initial concentration of fraction 2 and did not result from leakage of the hollow fibers. The loss is probably caused by irreversible adsorption of lipase to the fibers.

5) For the preparation shown in Fig. 1, most of the enzyme eluted in peak II. However, in other preparations the relative amounts varied, depending in part on the starting material; for eight ultrafiltrates chromatographed on DEAE-cellulose the average amount of protein recovered in peak I fractions was 39 ± 9 mol% of peaks I and II combined.

6) The colipase content of peak II enzyme was approximately 1:1 in all preparations. As shown in Fig. 1, the colipase in peak I fractions is due to overlap with peak II. Thus, the colipase content of the combined fractions from peak I is greatly influenced by the choice of the particular fractions used. For the eight preparations chromatographed on DEAE the mol % of colipase in the combined fractions from peak I was in one case 4 and in all others less than 2.

Chemical characterization

Samples (approximately 1 mg) of lyophilized colipase-free lipase and the lipase–colipase complex were analyzed for total fatty acids as described under Materials and Methods. There was, at most, 0.05% fatty acid by weight (about 10% on a mole basis) in each.

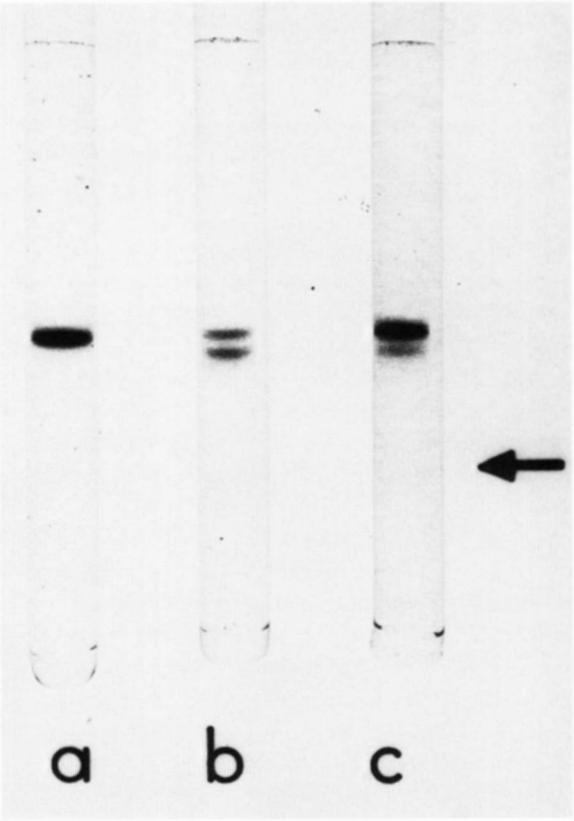

Fig. 2. Polyacrylamide gels of the lipase from peaks I and II of a DEAE-cellulose column of the same type as described in Fig. 1. (*a*) 12 μg of I, (*b*) 12 μg of II, (*c*) 9 μg of I plus 9 μg of II. The cathode (−) was at the top of the gels. The arrow points to the location of the colipase band originally visible in gels *b* and *c*.

Lyophilized samples of lipase from several preparations were also analyzed for total and lipid-soluble phosphate. No lipid-soluble phosphate could be detected (<3 mol %) but some phosphate was present in the total sample. It was shown that this phosphate was not derived from either DFP (18) or the phosphate buffer. These observations, together with the range of values of 0.4–10 mol/mol of enzyme, suggest that the phosphate is inorganic phosphate and is derived from the starting material. We did not observe any relationship between phosphate content and the kinetic and physical properties of lipase obtained.

The taurodeoxycholate content of a sample of peak II lipase was 13 mol %. Since the spectrophotometric assay employed is not specific for taurodeoxycholate but also responds to protein, this value must be considered an upper limit for bile salt content. It is reasonable to expect little or no bile salt contamination since we have observed that taurodeoxycholate shows a high affinity for DEAE-cellulose under the conditions employed for chromatography.

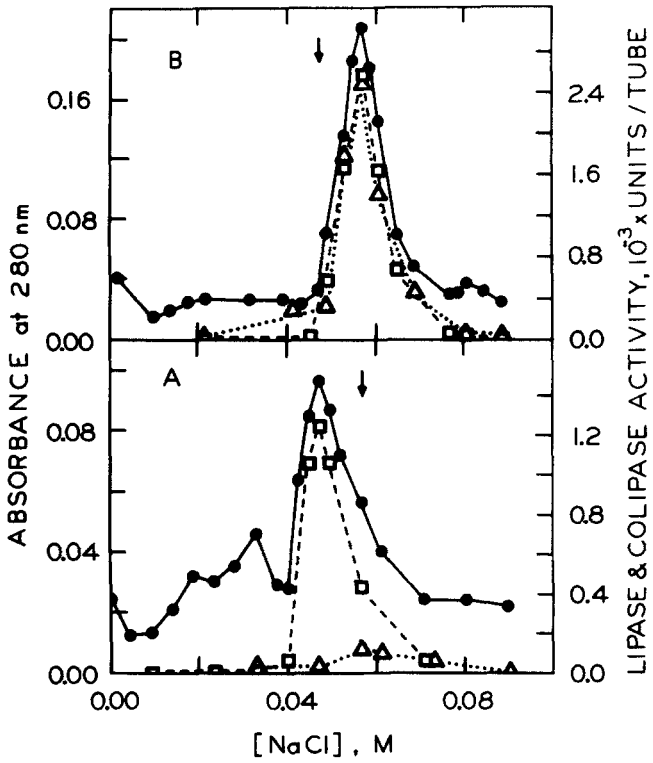

Fig. 3. Elution profiles from DEAE-cellulose chromatography after differential desorption of lipase from 400 g of beads. Procedure as described in the text except where noted below. *A*, Bottom figure. Initial desorption using 7 mM taurocholate in place of taurodeoxycholate in buffer C. Column dimensions, 0.6 × 19 cm; 100-ml linear gradient, 0–0.1 M NaCl in buffer; flow rate, 13 ml/hr; fraction volume, 2.2 ml; 4°C. ● —— ●, Absorbance at 280 nm; □ - - - □, lipase activity against tributyrin; △ ··· △, colipase activity. *B*, Top figure. Subsequent desorption with buffer C containing 3.5 mM taurodeoxycholate. The conditions and symbols are described in 3*a* above, except that the fraction volume was 2.4 ml. For comparison, the arrow in each figure indicates the position of the lipase peak obtained when desorbed from the bead surface with the other bile salt.

Kinetic parameters

The catalytic activity and interfacial stability of a sample of peak I lipase was measured in the tripropionin–siliconized glass bead assay system as described previously (8). Values of 3.5×10^3 S^{-1} and 5.7×10^{-3} S^{-1} were obtained at pH 7.5 for k_e, the pseudo first-order rate constant for catalysis, and k_i, the pseudo first-order rate constant for inactivation, respectively. These agree quite well with the values 3.2×10^3 S^{-1} and 5.5×10^{-3} S^{-1} reported (8) for lipase B purified according to Verger et al., as described in the Materials section. These two parameters were also measured as a function of pH in the absence of TDC, yielding profiles that were essentially identical to those previously reported (8).

The interaction of lipase and colipase in the presence of TDC was also examined as previously described (19). From a plot of the acceleration rate constant, k_{exp}, vs. the moles of colipase, the forward and reverse rate constants, k_{34} and k_{43}, respectively, were calculated to be 1.5×10^9 cm^3 S^{-1} mol^{-1} and 3.3×10^{-3} S^{-1}. These values compare favorably with the values 1.3×10^9 cm^3 S^{-1} mol^{-1} and 3.8×10^{-3} S^{-1} (19) obtained for lipase B purified by the method of Verger et al. (3).

Bile salt specificity

Taurodeoxycholate was used for the purification because we and others have previously demonstrated the ability of that bile salt to inhibit the interaction between lipase and substrate or apolar surface (8, 20, 21). We also examined the ability of a related bile salt, taurocholate, to function in the purification and obtained markedly different results. In a preliminary adsorption experiment with TC, the lipase obtained in fraction 2 was relatively colipase free. Therefore, in a subsequent purification, we eluted protein from the beads first with taurocholate and then with taurodeoxycholate. The concentration of TC employed in buffers A, B, and C was twice that for TDC because the CMC for TC is approximately twice that of TDC (20). Each of the eluates was concentrated by filtration as described above and chromatographed on a 20-cm DEAE-cellulose column in the usual manner. The elution profiles from these columns are shown in **Fig. 3,** *a* and *b*. Taurocholate eluted lipase essentially free of colipase whereas taurodeoxycholate subsequently eluted the lipase–colipase complex. The sodium chloride concentration at each peak agrees well with the data from TDC preparations of the type shown in Fig 1. The TC peak eluted at 0.048 M, corresponding to peak I lipase which elutes at 0.043 ± 0.003 M (SD). The TDC peak eluted at 0.059 M, corresponding to the colipase–lipase peak (II) which elutes at 0.057 ± 0.004 M (SD).

DISCUSSION

Using the method described above, porcine pancreatic lipase can be obtained in milligram quantities. By taking advantage of specific interactions between lipase, colipase, and bile salts in a batch procedure it is possible to eliminate several of the batch and column procedures previously required and to reduce purification time from 1–2 weeks to 24 hr while maintaining comparable yields (3, 6).

The product is obtained in two fractions from DEAE-cellulose. The first is lipase B which contains only small amounts of colipase and the second consists of lipase A and B with approximately equimolar amounts of colipase. In contrast, conventional pro-

cedures produce a lipase that contains 0–30 mol % colipase (4).

Chemical analyses showed that the products were essentially free of contaminating fatty acids, lipid-soluble phosphate, and taurodeoxycholate, although some phosphate, presumably inorganic, was found.

The kinetic properties of the relatively colipase-free product were identical to those of authentic lipase B, both with respect to the lipase alone and in its interactions with authentic colipase. Together, the characterization data indicate that the method yields a product that is essentially identical in its physical and kinetic properties to lipase prepared by conventional procedures (3, 6) but greatly reduces the number of steps and the time required.

The existence of a bile salt specificity showed that elution of the lipase–colipase complex from the beads is not a simple detergent effect. Whereas both forms of lipase are eluted by TDC, only the relatively colipase-free species is eluted with TC. Under our conditions TC micelles have an aggregation number of approximately 5 whereas TDC micelles contain 15–20 monomers (22). Thus, the concentration of TC micelles was about 6 times that of TDC micelles, indicating that the observed specificity is not simply a mass action effect, but is more likely related to the size of the micelles.

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