Properties of Serum Lipase in Patients with Various Pancreatic Diseases

Analysis by a New Serum Lipase Assay Method (The BALB-DTNB Method) in Combination with Gel-Filtration and Iso-Electrofocusing Techniques

Shigeru KUROOKA* and Tsugio KITAMURA**

*Research Laboratories, Dainippon Pharmaceutical Co., Ltd., Enoki-cho, Suita, Osaka 564, and **Section of Hepato-Pancreatic Diseases, The Center for Adult Diseases, Higashinari-ku, Osaka, Osaka 573

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Very low levels of lipase can easily be measured by a new serum lipase assay method (the BALB-DTNB method), using BAL-tributyrate (BALB) as a substrate, 5,5′-dithiobis(2-nitrobenzoic acid) as a chromogenic SH reagent, phenylmethylsulfonylfluoride as an inhibitor of esterases and sodium dodecyl sulfate as a surfactant. The BALB-DTNB method has a higher sensitivity than the conventional serum lipase assay methods, and proved useful for analyzing the properties of serum lipases in combination with gel-filtration on a Sephacryl S 200 column and isoelectrofocusing in an Ampholine column. Serum samples containing high levels of lipases from patients with pancreatic diseases or patients in whom the pancreatic exocrine gland had been stimulated by injecting caerulein and secretin were analyzed by these methods. The lipolytic profiles obtained indicated the presence of a lipase with an estimated molecular weight of 46,000 and isoelectric points of 7.4, 6.8, or/and 6.4. A lipase with properties similar to those of the serum lipase was found to be present in human pancreatic juice.

As a result of recent progress in studies on the properties of pancreatic lipase [EC 3.1.1.3] and the improvement of assay methods for the enzyme, human serum pancreatic lipase has been re-evaluated as a useful index, like serum amylase [EC 3.2.1.1], for the diagnosis of pancreatic diseases (1, 2).

We have developed (3, 4) a simple colorimetric method for serum lipase assay using an artificial substrate, BAL-tributyrate (BALB), in combination with a chromogenic SH reagent (Ellman's reagent, 5,5′-dithiobis(2-nitrobenzoic acid, DTNB) (5), an inhibitor of serum esterases (phenylmethylsulfonylfluoride, PMSF) (6) and a lipase activator (sodium dodecyl sulfate, SDS). This method (hereafter designated as the BALB-DTNB method) was found to be sensitive enough to measure the very low levels of lipase in the fractions separated after subjecting serum samples directly to gel-filtration on a Sephacryl S 200® column or to isoelectrofocusing in an Ampholine® column.

In view of the importance of confirming the specificity of the BALB-DTNB method for the assay of serum lipase originating from the pancreas, the gel-filtration and isoelectrofocusing profiles of the lipolytic activity of serum samples from various patients were compared with those of the pancreatic juice lipase.
MATERIALS AND METHODS

Reagents for the BALB-DTNB method were the same as those described in the previous papers (3, 4). Other reagents used in these experiments were all of reagent grade purchased from commercial sources.

Samples used for the lipase assay; Fresh human blood serum samples with elevated lipase activity were from patients either diagnosed as having pancreatic diseases or challenged by the pancreatic exocrine evocation test by injecting caerulein (i.m. 0.1 μg/kg) and secretin (i.v. 1 U/kg) (7). Pure pancreatic juice without bile contamination was obtained by canulating a drainage tube into the pancreatic main duct from patients that had received partial pancreatectomy due to cancer (8). Pancreatic juice containing bile was obtained through a drainage tube from the duodenum of patients subjected to the caerulein-secretin test (C.S. test). These samples were kept frozen at -20°C for several days to about one month before analysis.

Lipase Assays—1) BALB-DTNB method (3): The method has been developed into a convenient kit form (Lipase Kit “MARUPI,” Dainippon Pharmaceutical Co., Ltd., Osaka), consisting of the following four reagents;

a) Substrate solution (BALB)—20 mM BALB-20 mM SDS in ethanol.
b) Esterase inhibitor (PMSF)—20 mM PMSF in ethanol.
c) Chromogenic reagent (DTNB)—powder.
d) Buffer solution (TRIS)—1 M Tris•HCl (pH 8.5).

The chromogenic buffer stock solution of 3 mM DTNB-0.1 M Tris•HCl (pH 8.5) was reconstituted by adding TRIS (2.4 ml) and then distilled water (22 ml) to the DTNB powder (30 mg) (for 100 assays). The working chromogenic buffer solution of 0.3 mM DTNB-0.1 M Tris•HCl (pH 8.5) was prepared by mixing one part of the chromogenic buffer stock solution, one part of TRIS, and 8 parts of water. The stock solution can be used several times when kept frozen, preferably in separate bottles.

Assay procedure: For one sample assay, pipette into round-bottomed glass test tubes (1.2×10 cm) I and II, both DTNB-Tris working solution (1 ml) and the human serum sample (50 μl). Add PMSF (20 μl) to I and II, and incubate them at 30°C for 5 min. Then add BALB (0.1 ml) to I, mix well, and immediately incubate I and II at 30°C. After incubation for 30 min, stop the reaction by adding acetone (2 ml) to I. Add acetone (2 ml) and BALB (0.1 ml) to II. After swirling I and II well, centrifuge at 2,000 × g for 10 min to remove insoluble materials. Measure the absorbance (A) of TNB anions in the clear supernatants of I and II against water at 412 nm in a cuvette of 1 cm light path. Avoid exposing the TNB anions to reflected sunlight that decomposes the anions.

Serum lipase activity is expressed in BALB units (3). One BALB unit is defined as the enzyme activity giving A of 0.001 (I-II) at 412 nm when the serum sample (50 μl) is incubated for 30 min at 30°C under the assay conditions described above. Multiplying by a factor of 0.14, the BALB unit can be converted into the international unit expressing the lipase activity as the number of liberated SH group of BAL/min/liter of serum.

For the assay of lipase in the fractions obtained by gel filtration and isoelectrofocusing of serum or pancreatic juice, DTNB-Tris working solution (1 ml) containing 20 μl of normal human serum is used, because the BALB-DTNB method developed for the assay of lipase in human sera requires the presence of serum proteins (especially albumin) in the enzyme reaction mixture (3). The fractionated sample (0.2 ml for the serum fractions, and 10 μl for the pancreatic juice fractions) is added only to I; the enzyme reaction is started by adding BALB (0.1 ml) to both I and II, and stopped after incubation for an appropriate time depending on the lipase activity, preferably before A_{412} (I-II) exceeds I. The lipase activity is expressed arbitrarily as A_{412} (I-II)/reaction time (min)/sample volume (ml).

2) Turbidimetry: The lipase activity in serum or pancreatic juice was measured with a Serum Lipase Set (Harleco Co., U.S.A.) based on turbidimetry (9). The decrease in turbidity was measured at 400 nm using a Hitachi 125 double-beam spectrophotometer with an automatic sample exchanger, a thermo-regulator, and a recorder for the rate assay.

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Other assay methods: Human serum albumin (HSA) (10), horseradish peroxidase (POX) [EC 1.11.1.7] (Sigma Chemicals, U.S.A.) (11) and pseudocholinesterase (PsChE) [EC 3.1.1.8] (5) were measured according to the methods given in the references.

Gel-filtration method: A serum sample (0.2 ml) was mixed with 0.5 M KCl-0.25 M Tris·HCl (pH 8.0) (50 μl) containing markers for the calibration of molecular weight, and applied to a Sephacryl S 200® (Pharmacia Fine Chemicals, Uppsala, Sweden) column (1 × 54 cm) well equilibrated with 0.1 M KCl-0.05 M Tris·HCl (pH 8). The sample in the column was eluted with the same buffer and fractions of 0.8 ml were collected.

Pancreatic juice (0.15 ml) was diluted with an equal volume of 0.2 M KCl-0.1 M Tris·HCl (pH 8) containing the calibration markers, and a 0.25 ml portion was applied to the column and fractionated as described above. These experiments were carried out in a cold room (5°C).

Isoelectrofocusing method: Essentially according to the directions of the manufacturer (LKB, Bromma, Sweden), a serum sample (0.4 ml) or pancreatic juice (5 ml) was applied in a 0–55% (W/W) sucrose gradient on an isoelectrofocusing column (110 ml). The final concentration of carrier ampholite (Ampholine® pH 3.5–10) was 1%. Columns with the cathode on the top and the anode at the bottom, and vice versa, were run at 300 volts for 48 or 72 h at 5°C, and then fractions of 42 drops (about 2 ml) were collected.

RESULTS

1) Relationship between Types of Disease and Serum Lipase Activities measured by the BALB-DTNB Method and by Turbidimetry—According to the BALB-DTNB method, 200 serum samples (male 125, female 75) from volunteers showed activity of 74±37 (average±SD) BALB units, in good agreement with the data reported by Morishita et al. (12, 13), whereas the samples from patients diagnosed as having acute, chronic pancreatitis, pancreatic carcinoma (head) or diseases related to obstruction or stricture of the pancreatic duct, or destruction of the pancreas frequently gave values of more than 150 BALB units (average±2 SD of normal levels) (Table I). Patients that had undergone total pancreatectomy due to cancer gave less than 10 BALB units. When the pancreatic exocrine gland was stimulated by the C.S. test (7), increase in serum lipase activity was observed in some patients with suspected pancreatic diseases, suggesting the usefulness of this test for the diagnosis of mild pancreatic diseases.

Elevated serum lipase was also detected in some patients with renal insufficiency, confirming the findings of several authors (13–17).

Eighteen serum samples with lipase activity ranging from 40 to 990 BALB units were randomly selected from various patients diagnosed as having pancreatic or renal malfunctions, and their lipase activities were measured with a commercial kit, the “Serum Lipase Set,” based on turbidimetry (9). As shown in Fig. 1, the values measured by both methods were well correlated, indicating that the BALB-DTNB method can be used, like conventional methods, for the diagnosis of pancreatic diseases (2).
TABLE I. Serum lipase activities of normal subjects and patients with various diseases. Figures in parentheses show serum amylase activity (Caraway units).

<table>
<thead>
<tr>
<th>Serum samples</th>
<th>Diagnosed status</th>
<th>Serum lipase activity BALB units</th>
</tr>
</thead>
<tbody>
<tr>
<td>volunteers (n = 200)</td>
<td>healthy in appearance</td>
<td>8 ± SD = 74 ± 37</td>
</tr>
<tr>
<td>volunteers (n = 5 pooled)</td>
<td>in good health</td>
<td>110*E</td>
</tr>
<tr>
<td>N.M.</td>
<td>advanced pancreatic carcinoma</td>
<td>9,000*A</td>
</tr>
<tr>
<td>Y.M.</td>
<td>renal insufficiency</td>
<td>1,400*B</td>
</tr>
</tbody>
</table>

Subjects challenged by the C.S. test

<table>
<thead>
<tr>
<th>Subjects challenged by the C.S. test</th>
<th>before</th>
<th>after</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 h</td>
<td>2 h</td>
</tr>
<tr>
<td>Y.N.</td>
<td>94</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>(146)</td>
<td>(122)</td>
</tr>
<tr>
<td>T.J.</td>
<td>215</td>
<td>237</td>
</tr>
<tr>
<td></td>
<td>(131)</td>
<td>(136)</td>
</tr>
<tr>
<td>M.Y.</td>
<td>22</td>
<td>295*D</td>
</tr>
<tr>
<td></td>
<td>(124)</td>
<td>(137)</td>
</tr>
<tr>
<td>I.Ws.</td>
<td>8</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>(145)</td>
<td>(145)</td>
</tr>
<tr>
<td>M.T.</td>
<td>97</td>
<td>175</td>
</tr>
<tr>
<td></td>
<td>(257)</td>
<td>(224)</td>
</tr>
<tr>
<td>H.A.</td>
<td>285</td>
<td>383*C</td>
</tr>
<tr>
<td></td>
<td>(231)</td>
<td>(210)</td>
</tr>
<tr>
<td>removal of carcinoma</td>
<td>15</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>(83)</td>
<td>(72)</td>
</tr>
<tr>
<td>N.A.</td>
<td>187</td>
<td>165</td>
</tr>
<tr>
<td></td>
<td>(85)</td>
<td>(85)</td>
</tr>
<tr>
<td>N.I.</td>
<td>48</td>
<td>388*G</td>
</tr>
<tr>
<td></td>
<td>(97)</td>
<td>(155)</td>
</tr>
<tr>
<td>I.Wa.</td>
<td>28</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>(170)</td>
<td>(138)</td>
</tr>
<tr>
<td>H.I.</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>(103)</td>
<td>(95)</td>
</tr>
</tbody>
</table>

* Samples (A–H) were used for isoelectrofocusing.

2) Gel Filtration and Isoelectrofocusing Profiles of Lipase Activity measured by the BALB-DTNB Method—In order to confirm that these serum lipolytic activities measured by the BALB-DTNB method and turbidimetry are really due to lipase of pancreatic origin, serum samples from various patients were subjected to gel-filtration on a Sephacryl S 200 column and to isoelectrofocusing in an Ampholine column, and the profiles of the lipase activity (=lipolytic activity) were compared with those of lipase in the pancreatic juice.

a) Gel-filtration profiles: Various serum samples with more than 200 BALB units of lipase activity were subjected to gel-filtration on a Sephacryl S 200 column. Figure 2 shows a typical example of elution profile of the serum lipolytic enzyme (=lipase) from a patient with pancreatic carcinoma. The lipase was eluted later than human serum albumin (HSA, M.W. 68,000) and slightly faster than horseradish peroxidase (M.W. 40,000). The M.W. of serum lipase was estimated to be about 46,000 as shown in Fig. 3 in good
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Fig. 2. Gel-filtration profiles of serum lipase on a Sephacryl S 200 column. Serum sample (A) (0.25 ml) from a patient (N.S.) with pancreatic carcinoma was subjected to gel-filtration and analyzed as described in "MATERIALS AND METHODS" except that Blue Dextran 2000 (B.D. 2000, M.W. 200,000, Pharmacia Fine Chemical) (2 mg) and POX (10 μg) were added to the serum sample. The eluted fraction volume (V₀) at which the highest levels of the markers and lipase appeared were estimated as shown in the figure. The concentrations of markers in the fractions were expressed in arbitrary units and their highest concentrations are depicted at the same level for simplicity.

Fig. 3. Estimation of lipase molecular weight by gel-filtration. The M.W. of serum lipase (LIP) was estimated by plotting the Vₑ/V₀ ratio of markers against their M.W. values. The open circle shows serum lipase. V₀, Elution volume of Blue Dextran 2000; Vₑ, elution volume of markers and lipase (Fig. 2).

agreement with that of human pancreatic juice lipase determined from its amino acid composition (18).

Similar elution profiles of the serum lipolytic activity could be obtained with serum samples from patients with acute or chronic pancreatitis, or with renal insufficiency.

Pancreatic juice from patients who had undergone partial pancreatectomy owing to pancreatic cancer was also subjected to gel-filtration, and elution profiles obtained were confirmed to be the same as those of serum lipase.

b) Isoelectrofocusing profiles: Serum samples with more than 200 BALB units from patients with pancreatic diseases were subjected to isoelectrofocusing in an Ampholine column. A typical isoelectrofocusing profile of serum lipase from a patient (A (N.M.) in Table I) with pancreatic cancer is shown in Fig. 4. The lipolytic activity was resolved into one major peak with pI 6.8 and two small peaks with pI 7.4 and 6.4. The lipolytic activity of the major peak was also confirmed turbidimetrically using olive oil as a substrate, but the activities of the other two small peaks were too low to measure by turbidimetry.

Similar isoelectric resolution of the lipolytic enzyme was carried out for serum samples (C-H in Table I) from various patients challenged by the C.S. test (7), and the lipolytic profiles are summarized schematically in Fig. 5. In general, the lipolytic activity in the serum samples could be
Fig. 5. Isoelectrofocusing profiles of human serum lipase from various patients. The serum samples (B-H in Table I) were analyzed as described in the legend to Fig. 4. The enzyme activities were expressed as $A_{412}/0.2$ ml of the fraction/60 min for the lipase, and $A_{412}/$min/0.1 ml of the fraction for PsChE. $A_{280}$ represents serum proteins.

resolved into one to three peaks with pI values of 7.4, 6.8, or/and 6.4. The serum sample (C) from a patient with pancreatic carcinoma gave a lipolytic profile similar to the sample (A) from the patient with pancreatic carcinoma, and to the sample (E) from patient with chronic pancreatitis. The sample (H) from a patient in the recovery stage after the onset of acute pancreatitis showed a profile similar to that of the sample (D) from a patient with chronic pancreatitis. The pooled sera (F) from normal subjects gave a profile having one peak with pI 7.4, similar to that of a sample (G) from a patient with stones in the bile common duct. The serum sample (B) from a patient with renal insufficiency was found to give a lipolytic profile similar to those of the samples A, C, and E. It is likely that no definite relationship exists between the isoelectrofocusing profiles of the lipolytic activity and the type of disease, serum samples with high lipase having a tendency to contain much lipase with pI 6.8 and serum samples with low lipase containing only lipase with pI 7.4.

The lipolytic activity in pure pancreatic juice without bile contamination from partially pancreatectomized patients with cancer, or in crude pancreatic juice with bile contamination collected from the duodenum was also resolved by isoelectrofocusing (Fig. 6). As observed with serum samples, the lipolytic enzymes with pI 7.4, 6.8, and 6.4 were also detected in the juice, but several other lipolytic activity peaks were also found to be present between pH 5 and 3. The lipolytic peaks detected by the BALB-DTNB method could also be measured by turbidimetry, but the ratio of the enzyme activity measured by the two methods (BALB-hydrolyzing activity/olive oil-hydrolyzing activity) tended to increase as the pI became higher, suggesting the possibility of substrate specificity among these lipases with different pI values. It was confirmed that these lipases with different pI all gave M.W. values of about 46,000 by the gel-filtration method.

**DISCUSSION**

Our BALB-DTNB method seems to be one of the few methods sensitive enough to measure the low activity of pancreatic lipase in human sera and
has many advantages over the existing methods (1) in that firstly, the enzyme reaction proceeds proportionally with the incubation time as a result of eliminating the liberated BAL in the form of BAL-TNB from the reaction system, secondly, the activity can be measured by simple colorimetry, and thirdly, the enzyme reaction can be observed during the incubation time. It is possible to measure very low serum lipase activity by merely prolonging the reaction time.

The values measured by the BALB-DTNB method and by turbidimetry correlated well (Fig. 1), but the latter method lacked sensitivity for the assay of the low lipase levels (less than 200 BALB units) in normal sera.

There are very few reports on the characterization of serum pancreatic lipase, so it was necessary to confirm that the serum lipolytic enzyme activity measured by the BALB-DTNB method is not due to other lipolytic enzymes in mammalian sera (21-25), but to the lipase of pancreatic origin. The serum samples with high BALB units were fractionated by gel-filtration or isoelectrofocusing, which is a useful technique for the characterization of various enzymes without producing physico-chemical changes in them.

The M.W. of the lipolytic enzyme measured by the BALB-DTNB method and the gel-filtration method was roughly estimated to be 46,000, as reported by Vandermeers et al. (18). A lipolytic enzyme with similar M.W. was confirmed to be present in the human pancreatic juice collected in an ice-cold test tube from the main duct of the pancreas. However, the juice collected at room temperature sometimes contained a lipolytic enzyme with an estimated M.W. 32,000 in addition to the enzyme with M.W. 46,000 (19). It seems likely that the lipase with M.W. 46,000 may be split by proteolytic enzymes in the juice to form the lipase with M.W. 32,000, as suggested by Morgan et al. (27) based on their experiments on the gel-filtration of lipases in rat pancreatic juice. Straube et al. (20) and Kimura et al. (8) also suggested the possible presence of a lipase with M.W. 32,000 in human pancreatic juice.

The isoelectrofocusing profiles of serum lipase showed the presence of three kinds of lipases with pI 7.4, 6.8, or/and 6.4 which were also confirmed to be present in human pancreatic juice. Lipases with pI 3.4-4.5 were also found to be present in the juice. Straube et al. (20) demonstrated the presence of five kinds of lipases with pI 3.5 (I), 4.7 (Ia), 5.2 (Ib), 6.5 (IIa), and 7.5 (IIb) in human pancreatic juice collected through the duodenum, and suggested that IIa and Ib, or IIIa and IIIb are in the form of monomer and dimer. De Caro et al. (28) estimated the pI of human pancreatic juice lipase to be 5.80 and 5.85 by polyacrylamide gel-electrophoresis. Desharnais et al. (29) estimated the pI of pure pancreatic juice lipase from the rat to be 6.8 by polyacrylamide gel-electrophoresing. Pure porcine pancreatic lipase (type IV, Sigma Chemical Co., U.S.A.) gave a pI of about 5 by the present isoelectrofocusing method.

Inconsistency of pI among various reported pancreatic lipase is probably partly due to enzyme aggregation or modification during the isolation and purification processes, or partly because the enzymes bind to lipids such as bile acids and phospholipids. There is also possibility of differences in pI among animal species and sources (eg. pancreatic juice, pancreatic tissues, or serum).

As regards both M.W. and pI, the serum lipolytic enzyme measured by the BALB-DTNB method is different from other mammalian serum lipolytic enzymes, such as lipoprotein lipase [EC 3.1.1.34] (21), phospholipase A [EC 3.1.1.4] (22, 23), hepatic carboxylesterase [EC 3.1.1.1] (24), arylesterase [EC 3.1.1.2], pseudocholinesterase [EC 3.1.1.8] (25), or cholesterol esterase [EC 3.1.1.13] (26).

The reason why abnormally high lipase activity is observed in some sera form patients with renal insufficiency, as pointed out by several authors (13-17), still remains obscure. The present data that abnormally high lipolytic enzyme levels are present in the serum from patients with pancreatic disorders strongly suggest that the serum lipolytic enzyme in cases of renal disorder originates from the pancreas. No appreciable lipolytic activity in urine with high amylase activity from patients with pancreatic disorders could be detected by the BALB-DTNB method, as pointed out by Kreuzer et al. (30) and Rick (31) using their improved serum lipase assay methods. The normal kidney may have an ability to inactivate
the serum lipase, whereas disorder of the renal function (in glomeruli) may trigger accumulation of the lipase in the blood (17).

In conclusion, the BALB-DTNB method is considered to be reliable for serum lipase assays on the basis of the following three experimental findings:

i) Serum samples with high BALB values were frequently found in patients diagnosed as having obstruction or stricture of the pancreatic main duct, or challenged by the C.S. test. The enzyme activity was greatly diminished (almost to zero) in patients that had undergone total pancreatotomy.

ii) The serum lipolytic activity measured by the BALB-DTNB method correlated well with that measured by the conventionally accepted turbidimetric method using olive oil as a substrate.

iii) The lipolytic profiles of serum samples obtained by using the BALB-DTNB method, the gel-filtration method and the isoelectrofocusing method resembled those of pancreatic juice lipase, the molecular weight and isoelectric points being estimated to be 46,000 and pI 7.4, 6.8, or/and 6.4, respectively.

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