Time-Resolved Immunofluorometric Assay of Trypsin-2 Complexed with α₁-Antitrypsin in Serum

Johan Hedström,1,3 Jari Leinonen,1 Vesa Sainio,2 and Ulf-Håkan Stenman1

We developed a sensitive time-resolved immunofluorometric assay (IFMA) for trypsin-2 complexed with α₁-antitrypsin (AAT). We used a trypsin-2-specific monoclonal antibody on the solid phase and a europium-labeled polyclonal antibody to AAT as tracer. The detection limit is 0.05 μg/L and the range of linearity extends to 100 μg/L. We compared the clinical utility of the trypsin-2–AAT assay with that of free trypsinogen-2 and amylase in serum by studying 120 healthy subjects, 29 patients with acute pancreatitis, 11 with extrahepatic biliary obstruction, and 34 with acute abdominal disorders of extrapancreatic origin. In patients with acute pancreatitis the median concentration of trypsin-2–AAT in serum was 59-fold that in healthy controls, 42-fold that in patients with biliary obstruction, and 33-fold that in patients with acute abdominal disorders of extrapancreatic origin. These differences are greater than those for trypsinogen-2 (19-, 20-, and 28-fold, respectively) and amylase (5.4-, 6.5-, and 5.4-fold, respectively). Compared with the assays of free trypsinogen-2 and amylase, our assay of trypsin-2–AAT improved the clinical specificity for acute pancreatitis by eliminating false-positive results in our control groups. Increased concentrations of trypsin-2–AAT and trypsinogen-2 were also observed in patients with chronic renal failure undergoing dialysis.

Indexing Terms: acute pancreatitis/trypsinogen/amylase/hemodialysis

Acute pancreatitis is a potentially lethal disease requiring rapid diagnosis. Determinations of amylase in serum or urine are routinely used for this purpose but the tests lack specificity. In addition, concentrations of amylase in acute pancreatitis may not be very high initially, and usually decrease rapidly, making it difficult to monitor the disease. Therefore, the use of other pancreatic enzymes, e.g., the proteolytic enzymes, has gained interest (1). Determination of one such enzyme, trypsinogen-2, has given promising results (2).

Trypsinogen-2, also called anionic trypsinogen, is a serine protease (28 kDa) secreted by exocrine cells of the pancreas (3). When trypsin, the activated form of trypsinogen-2, reaches the bloodstream, it is rapidly inactivated by the major trypsin inhibitors α₂-macroglobulin and α₁-antitrypsin (AAT) (4). AAT (58 kDa) is synthesized in the liver and is one of the main protease inhibitors in blood (5–7). In severe pancreatitis the release of trypsin is thought to exceed the inhibitory capacity within or around the pancreatic gland, with potentially harmful effects (8).

Because activation of trypsinogen is a key feature of pancreatitis, one may assume that trypsin–inhibitor complexes are formed in this condition. Complexes between trypsin-1 and AAT have been evaluated recently (9), but to our knowledge complexes with trypsin-2 have not been studied. We have developed a sensitive time-resolved immunofluorometric assay (IFMA) for trypsin-2 complexed with AAT, and have studied the concentrations of this complex in sera from healthy people, patients with acute pancreatitis, patients with extrahepatic biliary obstruction, and patients with acute abdominal disorders of extrapancreatic origin. We compared the results for the trypsin-2–AAT complex with measurements of uncomplexed trypsinogen-2 and amylase.

Materials and Methods

Samples

Serum samples were obtained from 29 patients with acute pancreatitis, 11 patients with extrahepatic biliary obstruction, and 34 patients with acute abdominal disorders of extrapancreatic origin (Table 1). All samples were drawn within 24 h of the patients' admission to the emergency ward and before initiation of therapy. The diagnosis of acute pancreatitis was based on clinical and laboratory findings (measurements of serum and urine amylase and C-reactive protein) and the Ranson criteria (10), and was confirmed in all cases by a contrast computer tomographic scan of the pancreas. The diagnoses of extrahepatic biliary obstruction and of other acute abdominal disorders of extrapancreatic origin were based on regular criteria (clinical and laboratory methods, ultrasonography, and radiological examinations or operative findings). Gastric and duodenal ulcers as well as esophagitis and gastritis were diagnosed by endoscopy. Serum samples were also obtained from 15 patients with chronic renal failure on dialysis treatment. Serum samples from 120 blood donors were obtained from the Finnish Red Cross Blood Bank. All samples were stored at −20°C until assayed.

The procedures followed were in accordance with the Helsinki Declaration of 1975.

Materials

Reagents. The polyclonal antiseraum against AAT was obtained from Dakopatts (Glostrup, Denmark). The production of the monoclonal antibodies against trypsinogen-2 was described earlier (2).

1 Department of Clinical Chemistry, and 2 Department of Surgery, Helsinki University Central Hospital, SF-00290 Helsinki, Finland.
3 Author for correspondence. Fax Int + 358-0-4714804.
4 Nonstandard abbreviations: IFMA, immunofluorometric assay; AAT, α₁-antitrypsin; and ROC, receiver-operating characteristic.

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Buffers and reagent solutions. The assay buffer in the IFMA was 50 mmol/L Tris-HCl, pH 7.7, with 9 g/L NaCl (Tris-buffered saline) containing, per liter, 5 g of bovine serum albumin, 0.15 g of bovine globulin, and 0.5 g of NaN₃. The wash solution contained, per liter, 9 g of NaCl, 0.5 g of NaN₃, and 0.2 g of Tween-20. Enhancement solution was from Wallac Biochemical Laboratories (Turku, Finland).

Calibrators. Trypsinogen-2--AAT was prepared from pure human AAT (Sigma Chemical Co., St. Louis, MO) and trypsin-2, purified as described (2). Trypsinogen-2 was autodigested at 37°C for 2 h and then incubated for 16 h at 20°C with a sevenfold molar excess of AAT in IFMA assay buffer. The incubation mixture was separated by gel filtration and the concentrations of trypsinogen-2 and trypsinogen-2--AAT in the fractions were estimated by a trypsinogen-2 IFMA. Incubation with AAT reduced the trypsinogen-2 immunoreactivity to 6% of that in the control incubated with aprotinin. On this basis, we assumed that 94% of trypsinogen-2 had complexed to AAT. This preparation was used for calibration of the trypsinogen--AAT assay. Calibrators were prepared by diluting the complex with assay buffer to contain trypsinogen-2--AAT at concentrations of 0.1, 0.5, 1.0, 10, and 100 µg/L.

Procedures

Gel filtration. Fractionation of the trypsinogen-2--AAT standard was performed by gel filtration on a 1 × 30 cm column of Superdex 200 HR 10/30 (Pharmacia, Uppsala, Sweden) eluted with Tris-buffered saline. The flow rate was 30 mL/h, and 500-µL fractions were collected. The collection tubes were prefilled with 50 µL of assay buffer containing aprotinin (1.0 mg/L) to prevent proteolysis and nonspecific adsorption to the tubes. The elution volumes of IgG (150 kDa) and albumin (69 kDa) were used for a rough calibration of the column.

Time-resolved IFMA of trypsinogen-2 and trypsinogen-2--AAT. Trypsinogen-2 was determined as described (2). For assay of trypsinogen--AAT, a monoclonal antibody to trypsin-2 (14F10) (2) was coated onto microtiter wells and a polyclonal rabbit antibody to AAT (Dako, Glostrup, Denmark) labeled with a europium chelate (11) was used as tracer. Sample and assay buffer (25 and 200 µL, respectively) were pipetted into the coated wells. After incubation for 1 h the wells were emptied, washed twice with wash solution by an automated washer (Delfia Platewash 1296-024; Wallac), and filled with 200 µL of assay buffer containing 200 ng of tracer antibody. After further incubation for 1 h the wells were emptied and washed four times. Enhancement solution (200 µL) was added to each well, and after 5 min the fluorescence was measured with a 1234 Delfia Research Fluorometer (Wallac).

Amylase assay. Amylase was measured by an enzymatic colorimetric test in an automated analyzer (Hitachi 705E with SYS 1 α-Amylase EPS reagents from Boehringer Mannheim, Mannheim, Germany). The method was calibrated according to the Scandinavian Committee on Enzymes (12). The reference values of the method are 70–300 U/L (median 180 U/L).

Statistics. The detection limit of the assay was defined as the concentration corresponding to the fluorescence signal of assay buffer + 2 SD (calculated from 12 replicates). The reference range was determined on the basis of the 2.5 and 97.5 percentiles in sera from 120 blood donors. Analytical recovery was measured by adding 100 µg/L trypsinogen-2--AAT calibrator to human serum containing 8.8 µg/L trypsinogen-2--AAT. Recovery of the added amount of trypsinogen-2--AAT was measured by IFMA.

The ability of the various analyses to differentiate between pancreatitis and nonpancreatic disease was estimated by receiver-operating characteristic (ROC) curve analysis (13).

Results

Fractionation by gel filtration indicated that the trypsinogen-2--AAT standard contained 6% free trypsinogen-2. The IFMA for trypsinogen-2--AAT does not recognize free trypsinogen-2. The cross-reaction of trypsinogen-2--AAT in the trypsinogen-2 IFMA is 3.3% (molar basis) (Fig. 1). The dose–response curve for the trypsinogen-2--AAT IFMA shown in Fig. 2 reflects subtraction of the background.
radioactivity (2000 counts/s). The detection limit is 0.05 μg/L and the calibration curve is linear to 100 μg/L. The within-run CVs for trypsin-2–AAT were 6.4% and 4.8% at trypsin-2–AAT concentrations of 3.0 and 84 μg/L, respectively. The between-run CVs were 7.3% and 10.4% at 3.6 and 24 μg/L, respectively. Analytical recovery of trypsin-2–AAT added to human serum was 89–95% when trypsin-2–AAT was added to increase the concentrations by 9–45 μg/L.

To estimate the clinical accuracy of the assay, we determined the concentrations of trypsin-2–AAT, trypsinogen-2, and amylase in samples from patients with acute pancreatitis, patients with extrahepatic biliary obstruction, and patients with acute abdominal disorders of extrapancreatic origin (Table 2). The reference range for trypsin-2–AAT was 2.3–12 μg/L (median 4.2 μg/L) and that for trypsinogen-2 was 18–90 μg/L (median 39 μg/L). In patients with acute pancreatitis trypsin-2–AAT and trypsinogen-2 were increased substantially. The median concentration of trypsin-2–AAT was 59-fold that in healthy control subjects; that of trypsinogen-2 was 19-fold that in the controls; and that of amylase, 5.4-fold (Table 2).

There was no overlap in the values for trypsin-2–AAT between the patients with acute pancreatitis and the control groups. In comparison, 4 patients (14%) had values of trypsinogen-2, and 14 patients (48%) had values of amylase within the range of the values of the controls (Fig. 3).

The area under the ROC curve was 1.00 for trypsin-2–AAT, 0.996 for trypsinogen-2, and 0.929 for amylase, indicating that sensitivity and specificity were greater for trypsin-2–AAT than for the other analytes (Fig. 4).

To study whether increased concentrations of trypsin-2–AAT complex and trypsinogen-2 could be caused by chronic renal failure, we measured serum samples from 15 patients undergoing dialysis (Table 3). In the hemodialysis patients the median concentrations of trypsin-2–AAT were 11-fold that in patients undergoing peritoneal dialysis and 23-fold that in healthy controls.

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**Table 2. Median value and range of trypsin-2–AAT, trypsinogen-2, and amylase in serum of healthy controls and of different patient groups.**

<table>
<thead>
<tr>
<th></th>
<th>Trypsin-2–AAT, μg/L</th>
<th>Trypsinogen-2, μg/L</th>
<th>Amylase, U/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute abdominal disorders</td>
<td>Median: 7.6, Range: 2.4–36</td>
<td>Median: 27, Range: 7.8–145</td>
<td>Median: 178, Range: 78–711</td>
</tr>
</tbody>
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* Reference range.

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**Fig. 3.** Concentration of (A) trypsinogen-2 and (B) trypsin-2–AAT in serum samples from 120 healthy control subjects, 11 patients with extrahepatic biliary obstruction, 34 patients with acute abdominal disorders of extrapancreatic origin, and 29 patients with acute pancreatitis.

The concentration of serum amylase in the same patients is shown in (B). Short horizontal lines indicate the upper reference limits.
corresponding figures for trypsinogen-2 were 11- and 26-fold.

Discussion

We have shown that the concentration of trypsin-2-AAT complex in serum is a clinically more sensitive marker of acute pancreatitis than is the concentration of trypsinogen-2 on amylase. The larger increase in trypsin-2-AAT compared with trypsinogen-2 may indicate that in pancreatitis a higher proportion of trypsinogen-2 released into circulation has been prematurely activated, resulting in the formation of trypsin-2-AAT; alternatively, the trypsin-2-AAT complex may be metabolized more slowly. Premature activation of pancreatic enzymes is considered a cause of many of the severe complications of acute pancreatitis (14). Elimination of the inhibitors through complex formation may be a factor contributing to a fatal outcome (15–17). Therefore, measurement of complexed forms of trypsin may reflect the pathological changes at various stages of pancreatitis.

Trypsin-2-AAT occurs normally in circulation at concentrations only 5–20% of those of trypsinogen-2, whereas the concentrations of trypsin-1-AAT complexes are ~50% of those of trypsinogen-1. Patients with pancreatic disease have increased concentrations of trypsin-1-AAT, but measurement of trypsin-1-AAT has not been considered clinically useful because high concentrations of this complex are frequently found in patients with gastric ulcer and hepatobiliary obstruction, leading to false-positive results (9, 18, 19).

In healthy subjects the concentrations of trypsinogen-1 in serum are higher than those of trypsinogen-2, but in patients with pancreatitis the concentration of trypsinogen-2 increases more than that of trypsinogen 1. Thus trypsinogen-2 better differentiates between patients with pancreatitis and control subjects than does trypsinogen-1 (2, 20, 21). The present study shows that trypsin-2-AAT gives an even better separation than trypsinogen-2, which is clearly better than amylase. This was demonstrated by the values for the area under the curve in the ROC plots. With trypsin-2-AAT the separation between patients with acute pancreatitis and those with other acute abdominal disorders of extrapancreatic origin was complete.

In patients with renal failure undergoing hemodialysis or peritoneal dialysis, the concentrations of both trypsinogen-2 and trypsin-2-AAT were greatly increased. This suggests that trypsinogen is largely removed by the kidneys, which is to be expected on the basis of its relatively low molecular mass, ~25 kDa. The false increase has to be considered when one is evaluating results obtained by these assays.

Recent developments in immunoassay technology have made it possible to perform somewhat sophisticated assays fully automatically, even as stat-analyses. Once rapid automated methods are available, trypsin-2-AAT could be routinely used as a specific marker for pancreatic disease.

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References

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