Specific Immunoassay Reveals Increased Serum Trypsinogen 3 in Acute Pancreatitis

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BACKGROUND: Trypsinogen 3 is a minor trypsinogen isoform in the pancreas. In contrast with trypsin 1 and 2, trypsin 3 degrades pancreatic secretory trypsin inhibitor, which may lead to an excess of active trypsin and acute pancreatitis (AP). We developed an immunoassay for trypsinogen 3 and studied whether an assay of serum trypsinogen 3 is of clinical utility in the diagnosis of AP.

METHODS: Monoclonal antibodies were generated using recombinant human trypsinogen 3 as the antigen and used to establish a sandwich-type immunoassay. We analyzed serum trypsinogen 3 concentrations in 82 patients with AP and 63 patients with upper abdominal pain (controls). The reference interval was determined using serum samples from 172 apparently healthy individuals.

RESULTS: The measuring range of the trypsinogen 3 assay was 1.0–250 μg/L. Intra- and interassay CVs were <11%, and cross-reactivity with other trypsinogen isoenzymes was <0.1%. The median trypsinogen 3 concentration in serum from healthy individuals was <1.0 μg/L, and the upper reference limit was 4.4 μg/L. We observed increased trypsinogen 3 concentrations in patients with mild (median 9.5 μg/L) and severe (15.0 μg/L) AP; in both groups, the concentrations were significantly higher than in controls (median <1.0 μg/L) (P < 0.0001). In ROC analysis, the area under the curve of trypsinogen 3 for separation between AP and controls was 0.90 (P < 0.0001).

CONCLUSIONS: We established for the first time a specific immunoassay for trypsinogen 3 using monoclonal antibodies. Patients with AP were found to have increased serum concentrations of trypsinogen 3. The availability of this assay will be useful for studies of the clinical utility of trypsinogen 3.

Three human trypsinogen genes (PRSSI, PRSS2, and PRSS3) encode highly similar proteins, trypsinogen 1, 2, and 3, also called cationic trypsinogen, anionic trypsinogen, and mesotrypsinogen (1). The gene encoding trypsinogen C (TRY6) is regarded as a pseudogene. In the brain, the gene encoding trypsinogen 3 gives rise to an additional splicing variant, which encodes 2 trypsinogen forms (trypsinogen 4A and 4B) owing to alternative translation initiation (2). The activated form of trypsinogen 4 is identical to trypsin 3, and is hereafter referred to as trypsin 3.

Trypsinogens are produced at high concentrations by the pancreas. After secretion into the gastrointestinal tract, they are activated to trypsins (EC 3.4.21.4) by enterokinase (3). Trypsins are major digestive enzymes, and they further activate other pancreatic enzymes. Premature activation of trypsinogen within the pancreas is thought to play an important role in development of acute pancreatitis (AP) (4). In addition, trypsinogens are widely expressed outside the gastrointestinal tract. Several lines of evidence suggest that trypsins play a role in tumor invasion (1, 5). In ovarian cyst fluid, trypsinogen 2 concentrations are higher in malignant than in benign cysts (6). Many human cancer cell lines produce trypsinogen 2, which may be involved in the degradation of extracellular matrix, facilitating tumor invasion (7). High trypsin expression in gastric cancer cells is associated with increased tumorigenicity in nude mice (8).

Trypsin activates urokinase–type plasminogen activator and proforms of matrix metalloproteinase (MMP) 1, 2, 8, 9, and 13 in

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complexes with sin 1 and 2 enter into circulation, they rapidly form the diagnosis is based on clinical findings, determina-Because there are no pathognomic symptoms in AP, sis diagnosis of AP and neonatal screening of cystic fibro-nant growth 3 has been found to cleave CD109 and promote malig-generative disorders (14). Through activation of PARs, trypsin-has been shown to stimulate proliferation of cancer cells (15). Furthermore, PARs are involved in brain development and function, and they may mediate both neurodegeneration and neuroprotection in neurodegenerative disorders (14). In breast cancer cells, trypsin-3 has been found to cleave CD109 and promote malignant growth (16).

Measurement of trypsinogen is clinically useful for diagnosis of AP and neonatal screening of cystic fibrosis (1, 17). Symptoms of AP, a common disease, range from mild abdominal pain to organ failure and death. Because there are no pathognomic symptoms in AP, the diagnosis is based on clinical findings, determina-struction of serum amylase and lipase (18, 19), and computed tomography (20). The limitations of amylase and lipase measurements (18) have led researchers to search for more accurate tests. Trypsinogen 2 and trypsinogen activation peptide (17, 21, 22) have been found to improve diagnostics of AP. A rapid dipstick test for trypsinogen 2 has been shown to be a very ac-curate tool for diagnosis of AP (23). When active tryp-sin 1 and 2 enter into circulation, they rapidly form complexes with α2-macroglobulin (A2M) and α1-protease inhibitor (API) (1). When complexed with A2M, trypsin is not detectable by conventional immunoassays, but the trypsin-API complex can be specifically measured, and in AP its concentration in serum reflects activation of trypsinogen. We have developed specific assays for trypsinogen 1 and 2, and for trypsin 1 and 2 complexed with API (22, 24, 25), but assays for trypsinogen 3 have not been available.

The structures of the various trypsin isoenzymes are highly similar, with 87%–89% amino acid se-quence identity. Therefore, differentiation between them by immunological methods is challenging. Here we describe a specific sandwich-type immuno-assembly for trypsinogen 3 and show that serum trypsinogen 3 concentrations are increased in pa-tients with AP.

Materials and Methods

RECOMBINANT PROTEINS AND ANTIBODIES

Recombinant trypsinogen 1, 2, 3, and C with modified propeptide (MVPFDDDK) and N-terminal or C-terminal His-tags were produced in Escherichia coli and purified as described (12, 26). We used a synthetic gene (GenScript), cloned in pET28a(+) vector, to pro-duce trypsinogen 4B. We used QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene) to introduce a mutation into trypsinogen 3 cDNA, producing trypsinogen 3 containing the Lys23Gln mutation and an N-terminal His-tag. The Lys23Gln mutation in-creases the stability of trypsinogens by removing the cleavage site of the activation peptide (27). We ob-tained trypsinogen 3 by activating 2 μg recombinant trypsinogen 3 (without mutation) containing an N-terminal His-tag with 0.15 μg enterokinase (Roche Diagnostics) in 100 μL of 50 mmol/L Tris buffer, pH 8.0, containing 0.01% BSA, for up to 24 h at room temperature. To study the effect of inhibition on tryp-sin 3 immunoreactivity, we added 1.4 μL Pefabloc SC (final concentration 1.3 mmol/L, Roche Diagnostics), 20 μL A2M (final concentration 1.1 g/L, Athens Research), or 200 μL normal human serum (pooled from healthy volunteers) after 1- or 2-h activation to 40-μL aliquots of activated trypsin and incubated them for approximately 22 h. We measured the activity of tryp-sin 3 using the trypsin substrate S-2222 (Chromogenix Instrumentation Laboratory) as described (12). We generated mouse monoclonal antibodies (MAbs) against trypsinogen 3 according to a previously de-scribed protocol (22), using recombinant human trypsinogen 3 as antigen (12). Antibody clones reacting with trypsinogen 3 were selected using Ni-NTA–coated 96-well microtiter plates (Qiagen). Briefly, 1 μg trypsinogen 3 with a His tag was added per well in 100 μL assay buffer (50 mmol/L Tris·HCl, pH 7.7, containing 9 g NaCl, 5 g BSA, 0.1 g Tween 20, 8 mg diethylene triamine penta acetic acid, and 0.5 g NaN3 per liter) containing 10 mmol/L benzamidine. After 1 h incuba-tion and washing, 50 μL antibody containing cell cul-ture supernatant and 50 μL assay buffer were added for 1 h. The wells were washed, and 50 ng Eu-labeled rabbit antimouse antibody (Dako) in 200 μL assay buffer was added. After washing the wells and adding 200 μL enhancement solution (Perkin-Elmer), we measured fluore-scence with a time-resolved Victor2 V fluorometer (Perkin-Elmer).

SDS-PAGE AND WESTERN BLOTTING

Samples were boiled in reducing SDS sample buffer and separated by electrophoresis in 4%–12% NuPAGE Bis-Tris gels run in 2-(N-morpholino)ethanesulfonic acid (MES) buffer (Invitrogen), followed by silver
staining (28) or transfer of proteins to Immobilon-P Transfer Membrane (Millipore). The membrane was blocked by incubating the filter for 18 h at 4 °C with 1% BSA in 50 mmol/L Tris–HCl, pH 7.8, containing 9 g/L NaCl and 0.5 g/L NaN₃, and incubated overnight at 4 °C with mouse monoclonal anti-His₆ antibody (1:200 dilution, Roche Diagnostics), followed by detection with horseradish peroxidase–conjugated polyclonal rabbit anti–mouse immunoglobulin (1:1000 dilution, code P0260, Dako), using ECL Western blotting detection reagents (GE Healthcare).

IMMUNOFLUOROMETRIC ASSAY FOR TRYPsinOGEN 3
We measured trypsinogen 3 using a sandwich-type immunoassay using MAb F136–13E6 for capture and MAb F141–5F3 labeled with a Eu-chelate (Perkin-Elmer) as a tracer. The assay was performed essentially as described (22). Briefly, 25 μL sample or calibrators together with 200 μL assay buffer were incubated in microtiter plate wells coated with capture antibody (10 mg/L) for 1 h, followed by washing and addition of Eu-labeled tracer antibody (100 ng/well in 200 μL assay buffer) for 0.5 h. Calibrators containing 1.0–250 μg/L of trypsinogen 3 with the Lys23Gln mutation were prepared in assay buffer. We determined the content of trypsinogen 3 on the basis of the absorbance at 280 nm using an absorbivity of 1.6, based on ProtParam tool of ExPASy Proteomics Server (29). Intra- and interassay CVs of the assay were determined from 9 and 12 replicates, respectively, using serum samples containing 1.5–110.4 μg/L trypsinogen 3.

SERUM SAMPLES AND PATIENTS
We obtained serum samples from 172 apparently healthy subjects participating in the Nordic Reference Interval Project (NORIP) (30) and from 82 patients with AP admitted to Helsinki University Central Hospital between November 2002 and October 2004. The study protocol was approved by the Surgical Ethical Review Board of the Joint Authority for the Hospital District of Helsinki and Uusimaa. Informed consent was obtained from each patient or next of kin. The diagnosis of AP, made by experienced surgeons at the emergency ward upon arrival, was based on typical clinical findings (acute onset of epigastric pain, nausea, and vomiting), increased serum pancreatic amylase, and/or typical findings in computed tomography in 34 patients. Pancreatic amylase concentrations were measured with the Modular clinical chemistry analyzer (Hitachi) using Pancreatic α-Amylase liquid kit (Roche Diagnostics) according to the recommendations of the IFCC. The reference interval of the method (10–65 U/L) is based on NORIP (30). The cutoff value for diagnosing AP was 195 U/L, i.e., 3 times the upper reference limit, as widely accepted (17). The median delay from beginning of symptoms to hospital admission was 24 h (range 2–72 h). Patients with a delay of more than 72 h were excluded. The severity of AP was determined according to the Atlanta classification, i.e., patients with organ dysfunction and/or necrotic pancreas or pseudocysts were classified as severe AP patients (31). Serum samples for determination of trypsinogen 3 were collected on admission to hospital. The characteristics of the patients are shown in Table 1. Of the 25 patients with severe AP, 6 suffered from organ dysfunction due to respiratory and/or renal insufficiency. Twenty patients had recurrent AP. Sixty-three patients with upper abdominal pain of an etiology other than acute pancreatitis were used as a control group (see Supplemental Table, which accompanies the online version of this article at www.clinchem.org/content/vol57/issue11). The AP and control samples were stored at −20 °C before the measurement of trypsinogen 3 concentrations for 2.6–4.4 years (median 3.6) and 3.5–5.0 years (median 4.4), respectively. The stability of trypsinogen 3 was studied using 11 serum samples from AP patients. After the assay, these samples were stored for 3.7 years at −80 °C before reassy.

ANION EXCHANGE CHROMATOGRAPHY
Pooled serum (0.4 mL) from AP patients was applied to a PD-10 column (GE Healthcare) and eluted with 50 mmol/L Tris–HCl buffer, pH 8 (Tris buffer). The protein-containing fractions were pooled (3.5 mL) and

**Table 1. Characteristics and outcome of the patients with AP.**

<table>
<thead>
<tr>
<th>Character</th>
<th>Median (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, M/F</td>
<td>59/23</td>
</tr>
<tr>
<td>Age, years</td>
<td>50 (19–87)</td>
</tr>
<tr>
<td>Delay of symptoms, h</td>
<td>24 (2–72)</td>
</tr>
<tr>
<td>Etiology, n</td>
<td></td>
</tr>
<tr>
<td>Alcohol</td>
<td>47</td>
</tr>
<tr>
<td>Biliary</td>
<td>19</td>
</tr>
<tr>
<td>Other</td>
<td>16</td>
</tr>
<tr>
<td>Severity, n</td>
<td></td>
</tr>
<tr>
<td>Mild</td>
<td>57</td>
</tr>
<tr>
<td>Severe</td>
<td>25</td>
</tr>
<tr>
<td>Organ failure</td>
<td>6</td>
</tr>
<tr>
<td>APACHE IIa,b</td>
<td>6 (0–16)</td>
</tr>
<tr>
<td>SOFAb</td>
<td>2 (0–8)</td>
</tr>
</tbody>
</table>

* APACHE II, Acute Physiology and Chronic Health Evaluation II; SOFA, Sequential Organ Failure Assessment.

b At admission to hospital.
applied to a MonoQ anion exchange column (GE Healthcare) and eluted with a 30-min gradient of 0–500 mmol/L NaCl in Tris buffer. Flow rate was 0.5 mL/min and fractions were collected at 0.5-min intervals. Trypsinogen 1 and 2 concentrations were measured as described (22). These assays have <1% cross-reactivity with trypsinogen 3 (unpublished results).

DATA ANALYSES
Reference values were calculated separately for females and males and for age groups 18–30, 31–50, 51–70, and >70 years by use of Analyse-it for Microsoft® Excel 2003 (version 2.04, Analyse-it Software). We carried out statistical comparisons and correlation analysis between the groups using Mann–Whitney U-test and Spearman’s correlation analysis (SPSS Statistics 17.0, IBM). We performed ROC curve and linear regression analyses using Analyse-it. Results below the limit of quantification (1.0 μg/L) were assigned a value of 0.5 μg/L. P values <0.05 were regarded as statistically significant.

Results
IMMUNOFLUOROMETRIC ASSAY FOR TRYPsinogen 3
Ten antibodies were tested in all possible combinations in a sandwich-type immunoassay for trypsinogen 3. Some combinations gave no signal, whereas others also recognized trypsinogen 1 or 2. A specific assay for trypsinogen 3 was obtained using MAb F136–13E6 as capture antibody and F141–5F3 as tracer. The assay recognized both trypsinogen 3 and its activated form, trypsin 3. However, the responses of active trypsin 3 and trypsinogen 4B were 50% and 44%, respectively, that of trypsinogen 3, although no significant autolysis of trypsin 3 was detected by SDS-PAGE and silver staining (Fig. 1). Although inhibition of enterokinase-activated trypsin 3 with Pefabloc SC increased the immunoreactivity slightly, incubation of trypsin 3 with A2M or serum decreased it by 99%.

The calibration curve was linear over the range 1.0–250 μg/L (Fig. 2). Intraassay CVs were 9.9% and 7.6% at concentrations of 1.5 and 24.5 μg/L, respectively, and interassay CVs were 10.6% and 0.7% at concentrations of 15.0 and 110 μg/L. The cross-reactivity with recombinant trypsinogen 1, 2, and C was <0.1%. The recovery of recombinant trypsinogen 3 (6.4 and 25.8 μg/L) added to the serum containing 20.8 μg/L trypsinogen 3 was 81% (16%) and 110% (15%), respectively [mean (SD), n = 3 for both]. The lower limit of detection was 0.4 μg/L, as determined on the basis of the mean signal in the 0-calibrator plus 3 SD (n = 10), and the lower limit of quantification was 1.0 μg/L, as the lowest calibrator concentration with an interassay CV <20% (n = 5).

The specificity of the assay was determined after separation of the different trypsinogen isoenzymes in sera from AP patients by ion exchange chromatography. The concentrations of trypsinogen 1, 2, and 3 were determined in the fractions (Fig. 3). The trypsinogen 3 assay did not recognize the other trypsinogens (Fig. 3).
REFERENCE VALUES FOR TRYPsinogen 3

Reference intervals were determined using 172 serum samples from healthy individuals. No statistically significant differences in trypsinogen 3 concentrations were found between the sex or age groups comprising 10–28 subjects. We thus calculated common reference intervals for adults. Based on the 97.5 percentile, the upper reference limit was 4.4 g/L and the range 1.0–13.5 g/L. Of the samples, 55% contained 1.0 g/L trypsinogen 3.

EFFECT OF SAMPLE STORAGE

The stability of trypsinogen 3 was studied in 11 serum samples from patients with AP, containing 21–70 g/L trypsinogen 3. The recovery was 89% (23%) (range 28%–114%) after storage for 3.7 years at −80 °C.

TRYPsinogen 3 IN PATIENTS WITH AP

In patients with mild AP, the median concentration of trypsinogen 3 was 9.5 µg/L (95% CI 7.3–20.5), and in severe AP, 15 µg/L (5.5–20.7), being higher (P < 0.0001 for both) than in the control group with upper abdominal pain, <1.0 µg/L (<1.0–1.2) (Table 2, online Supplemental Fig.). Trypsinogen 3 concentrations were higher in alcohol-induced AP (median 17.4 µg/L, 95% CI 8.4–23.4) compared with biliary-induced AP (median 6.7 µg/L, 95% CI 2.1–9.5, P = 0.015), and in AP of other etiologies (median 12.9 µg/L, 95% CI 5.9–31.5) compared with biliary-induced AP (P = 0.037) (Table 2). Patients with recurrent AP showed higher concentrations of trypsinogen 3 (median 17.4 µg/L, 95% CI 8.4–23.4) compared with non-alcohol-induced AP (median 17.4 µg/L, 95% CI 8.4–31.5, P = 0.015), and in AP of other etiologies (median 22.9 µg/L, 95% CI 9.3–65.6) compared with biliary-induced AP (P = 0.043). There was no statistically significant difference in trypsinogen 3 concentrations between patients with mild and severe AP (P = 0.75). In ROC analysis, the area under the curve (AUC) of trypsinogen 3 for separation between AP and patients with upper abdominal pain was 0.90 (95% CI 0.85–0.95, P < 0.0001), in mild AP 0.88 (95% CI 0.82–0.94, P < 0.0001), and in severe AP 0.93 (95% CI 0.87–1.00, P < 0.0001) (Fig. 4). AUC of trypsinogen 3 in patients with recurrent AP was 0.94 (95% CI 0.86–1.01, P < 0.0001), compared to control group.

No relationship was found by linear regression analysis between serum trypsinogen 3 and amylase in AP (R² = 0.02, P = 0.169), mild AP (R² = 0.01, P = 0.372), severe AP (R² = 0.09, P = 0.136), and non-alcohol-induced AP (R² = 0.04, P = 0.264). On the

**Table 2. Clinical characteristics and laboratory values of patients with AP and controls.**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Trypsinogen 3, µg/L</th>
<th>C-reactive protein, mg/L</th>
<th>Amylase, U/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>All AP patients</td>
<td>82</td>
<td>12.0 (&lt;1.0–195)</td>
<td>20 (5–440)</td>
<td>1600 (110–19 500)</td>
</tr>
<tr>
<td>Severity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mild</td>
<td>57</td>
<td>9.5 (&lt;1.0–195)</td>
<td>14 (5–230)</td>
<td>2050 (110–19 500)</td>
</tr>
<tr>
<td>Severe</td>
<td>25</td>
<td>15.0 (&lt;1.0–114)</td>
<td>210 (5–440)</td>
<td>1100 (150–7730)</td>
</tr>
<tr>
<td>Etiology</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alcohol</td>
<td>47</td>
<td>17.4 (&lt;1.0–195)</td>
<td>33 (5–440)</td>
<td>1240 (120–6470)</td>
</tr>
<tr>
<td>Biliary</td>
<td>19</td>
<td>6.7 (&lt;1.0–45.1)</td>
<td>11 (5–430)</td>
<td>2630 (260–19 500)</td>
</tr>
<tr>
<td>Other</td>
<td>16</td>
<td>12.9 (&lt;1.0–89.7)</td>
<td>26 (5–350)</td>
<td>3290 (110–10 250)</td>
</tr>
<tr>
<td>Non-alcohol</td>
<td>16</td>
<td>8.4 (&lt;1.0–89.7)</td>
<td>14 (5–430)</td>
<td>3120 (110–19 500)</td>
</tr>
<tr>
<td>Recurrent</td>
<td>20</td>
<td>20.6 (&lt;1.0–133)</td>
<td>20 (5–440)</td>
<td>1360 (120–6040)</td>
</tr>
<tr>
<td>Non-recurrent</td>
<td>62</td>
<td>9.0 (&lt;1.0–195)</td>
<td>20 (5–430)</td>
<td>2040 (110–19 500)</td>
</tr>
<tr>
<td>Controls</td>
<td>63</td>
<td>&lt;1.0 (&lt;1.0–10.3)</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

a Data are median (range).

b ND, not determined.
Immunoassay for Trypsinogen 3

In contrast with trypsin 1 and 2, trypsin 3 does not form complexes with API or other common serpins in plasma (9–11, 13, 32). However, we found that addition of A2M or serum to trypsin 3 almost completely abolished its immunoreactivity, whereas the recovery of recombinant Lys23Gln trypsinogen 3 in serum was close to 100%. This suggests that, like trypsin 1 and 2, trypsin 3 forms complexes at least with A2M in circulation. Separation of serum from patients with AP by anion exchange chromatography showed that trypsinogen 3 eluted in 1 major and 2 minor peaks, suggesting that the assay measures, in addition to free trypsinogen 3, some minor trypsin 3 and/or trypsinogen 3 forms or complexes with inhibitors that remain to be identified. The alternative name of trypsinogen 3, mesotrypsinogen, refers to its isoelectric point between those of trypsinogens 1 and 2 (9). Therefore it is somewhat surprising that trypsinogen 3 elutes first in anion exchange chromatography. However, elution is not solely dependent on isoelectric point, as a high density of charged groups affects the behavior of proteins in ion exchange chromatography (33).

Altered expression or activation of trypsinogens is associated with several diseases, including cancer and AP (1, 5). Mutations in the PRSS1 gene encoding trypsinogen 1 (4) and in the SPINK1 gene that encodes pancreatic trypsin inhibitor PSTI are associated with increased risk of AP (1). Increased intrapancreatic trypsin activity may cause development of AP, which is associated with strongly increased serum concentrations of trypsinogen 2 and trypsin 2–API. These are sensitive serum markers for the disease, and trypsin 2–API correlates strongly with disease severity because it reflects activation of trypsinogen (1, 17, 21, 23, 24, 34).

Trypsinogen 3 represents only 4%–10% of total trypsin in the pancreas (35), but it may play a key role in the development of AP because of its ability to degrade PSTI (13). Therefore, analysis of trypsinogen 3 in serum is of potential diagnostic and prognostic value. We found significantly increased concentrations of trypsinogen 3 in serum from patients with AP. Analysis of ROC plots shows that trypsinogen 3, indeed, discriminates AP patients from controls with high accuracy. However, in this respect, trypsinogen 3 seems to be no better than trypsinogen 2 or amylase (17, 22, 24). Unfortunately, we were not able to compare whether trypsinogen 3 is a better marker than amylase for AP, owing to the lack of amylase determinations in the controls. Interestingly, among patients with AP of various etiologies, trypsinogen 3 correlated with amylase only in those with alcohol-induced disease. Thus it may reflect a different pathological mechanism than amylase. Furthermore, it should be noted that an increased amylase is one of the diagnostic criteria for diagnosis of AP. Obviously, elucidation of the role of trypsin 3

Discussion

The expression and functions of trypsin 3 differ from those of the other trypsins. Therefore, development of an immunoassay facilitating studies on the physiological role and diagnostic use of trypsin 3 in various diseases is potentially important. The present assay is highly specific and sensitive enough to measure trypsinogen 3 in clinical samples. Specificity of the assay was confirmed by analysis of 3 trypsinogen isoenzymes in serum fractionated by ion exchange chromatography and by analysis of recombinant trypsinogens.

The assay also detects activated recombinant trypsin 3, but recognition is about 50% of that for trypsinogen 3. Inactivation of trypsin 3 by Pefablock SC restores some of the immunoreactivity. This may be due to a conformational change, but also to inhibition of the tryptic activity, which may cause degradation of the antibodies used in the assay. It is also possible that the N-terminal propeptide or even the His tag are part of the epitope recognized by 1 of the antibodies used, and removal of these decrease the binding affinity. This is supported by the observation that both recombinant trypsin 3, i.e., trypsinogen 3 without propeptide and His-tag, and nonactivated trypsinogen 4B, which has a different N-terminal propeptide than trypsinogen 3, show reduced immunoreactivity compared to trypsinogen 3. Contrary to trypsin 1 and 2, trypsin 3 is not very susceptible to autolysis (13), as also shown here. Thus it is unlikely that autodegradation of trypsin 3 explains the reduced immunoreactivity.

In other hand, in alcohol-induced AP, serum trypsinogen 3 and amylase correlated significantly ($R^2 = 0.25, P = 0.0003$).

![Fig. 4. ROC curve of trypsinogen 3 for separation between acute pancreatitis and controls with upper abdominal pain not caused by pancreatitis.](image-url)
and/or trypsinogen 3 in AP and the influence of sample timing following the onset of AP needs further studies. To optimize management and prevention of recurrent pancreatitis, it is essential to identify its etiology. In the Western world, biliary stones and alcohol use are the main etiological causes (36), whereas in Finland, alcohol-induced AP is most common (37). Different laboratory tests have been used to identify the etiology of pancreatitis. Serum disialotransferrin is a sensitive marker for alcohol abuse, and it is also a promising marker for alcohol-induced AP (38). Furthermore, the ratio of trypsin 2–AP complex to trypsinogen 1 has been found to discriminate between biliary and alcohol-induced AP (39). In addition to identification of the etiology of AP, prediction of the severity of the disease at admission is a challenge. As severe AP is associated with organ dysfunction, multiorgan failure, and death (31), early identification of severe cases is important for proper treatment. In the present study, trypsinogen 3 did not predict the severity of AP, but it would be interesting to study whether complexes between trypsin 3 and some yet-unidentified inhibitors would reflect early events in the development of AP.

In conclusion, we have developed for the first time a specific and sensitive immunoassay for trypsinogen 3 and determined the reference interval. We showed that most patients with AP have clearly increased serum concentrations of trypsinogen 3. Trypsinogen 3 reflects pancreatitis in a different way than amylase and it may thus facilitate differential diagnosis of AP of various etiologies. As the physiological functions and the site of expression of various trypsinogens differ, this assay is likely to be useful for studies on trypsinogen 3 in other disorders.

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Immunoassay for Trypsinogen 3