Radioimmunoassay of Active Pancreatic Enzymes in Sera from Patients with Acute Pancreatitis. I. Active Carboxypeptidase B

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Previous studies have suggested that measurement of active enzymes in relation to proenzymes in serum of patients with pancreatitis may reflect the degree of zymogen activation in the gland. Here we describe the first single-tube assay for an active form of a pancreatic enzyme that is ordinarily synthesized as a proenzyme. Human procarboxypeptidase B, which we purified to near homogeneity, is approximately 13,000 Da larger than the active enzyme (EC 3.4.17.2). Antibodies specific for active carboxypeptidase B were obtained by affinity chromatography of anti-carboxypeptidase B antisera on a gel containing procarboxypeptidase B, then used to develop a single-tube radioimmunoassay for measuring active carboxypeptidase B in serum. Using this assay, we were able to detect, for the first time, active carboxypeptidase B in sera from patients with acute pancreatitis. Preliminary data show a correlation between the serum concentrations of active carboxypeptidase B and those of active trypsin complexed with serum inhibitors, but no correlation with serum amylase values.

Additional Keyphrases: proenzyme · comparison with trypsin and amylase activities · trypsin · trypsinogen · diagnosis and prognosis · procedures for enzyme purification, antibody production

Because inappropriate activation and subsequent release of pancreatic zymogens may be a major step in the pathogenesis of acute pancreatitis (1–4), it is remarkable that none of the existing assays for pancreatic enzymes in serum differentiates between active enzymes or proenzymes from the pancreas, including those for trypsin (5–8), chymotrypsin (9), elastase (10), or carboxypeptidase B (11). Pancreatic amylase (12) and lipase (12) are not synthesized as zymogens; therefore, assays of these enzymes provide no information on zymogen activation but merely reflect protein release from the pancreas.

Recent studies in our laboratory and others have demonstrated the presence of two forms of inhibitor-bound active trypsin (EC 3.4.17.2), and increased concentrations of trypsinogen in plasma from patients with acute pancreatitis (13, 14) and from dogs (15) and rats (16) with experimental pancreatitis, but not in plasma of healthy subjects (5, 6, 15, 16). Preliminary data suggest that the concentration of inhibitor-bound trypsin may better reflect the severity of the disease than do either the total immunoreactive trypsin in serum or amylase activity in serum (13, 14, 16).

The experimental methodology used to obtain these data consisted of chromatography of serum on size-exclusion columns, to separate trypsinogen and the various forms of inhibitor-bound trypsin, followed by radioimmunoassay of the resulting fractions of effluent—a procedure too laborious for clinical use. Our goal in the current study was to develop an assay for measuring concentrations of an activated pancreatic enzyme in serum by a single-tube procedure. Given the close structural homology between trypsin and trypsinogen (17), we thought it unlikely that an immunoaassay could distinguish the two. In contrast, bovine carboxypeptidase B reportedly differs from its zymogen by about 23,000 Da (18), which led us to hypothesize that antibodies specific for active human carboxypeptidase B (peptidyl-l-lysine-l-arginine) hydrolase; EC 3.4.17.2) might be used to measure the amount of active carboxypeptidase B in patients' sera. Because trypsin activates procarboxypeptidase B as well as trypsinogen, the activation of procarboxypeptidase B to yield carboxypeptidase B in plasma might parallel the activation of trypsinogen to yield trypsin.

Here we describe the purification of human procarboxypeptidase B, the purification of antibodies specific for the active enzyme by chromatography of anti-carboxypeptidase B antibodies on a procarboxypeptidase B affinity gel, and the development of an immunoaassay for active human carboxypeptidase B in serum. We also report the correlation between the concentrations of active carboxypeptidase B, amylase, and inhibitor-bound activated trypsin in serum.

Materials and Methods

Purification of Procarboxypeptidase B

We used procedures similar to those for purifying human carboxypeptidase B from pancreatic tissue (19). Only tissue obtained at autopsy within 12 h of death, from an individual considered to be previously healthy, was used for zymogen purification. The organ was either processed without delay or stored at −70 °C until used. The tissue was extracted with acetone and the extract evaporated under reduced pressure. The resulting powder was stored at −20 °C until used in subsequent steps, which were carried out at 2–4 °C.

We suspended 2.5 g of the dried powder in 50 mL of pH 7.2 buffer containing, per liter, 50 mmol of Tris HCl, 50 mmol of NaCl, 10 mmol of benzamidine, and 1.5 g of bovine pancreatic trypsin inhibitor (a kind gift from Bayer AG, Wuppertal, F.R.G.) or trypsin inhibitor from lima bean (Sigma Chemical Co., St. Louis, MO). After homogenizing the mixture for 1 min (VirTis "45" homogenizer; VirTis Corp, Gardiner, NY), we stirred it for 1 h, centrifuged (17,000 × g, 15 min), and applied the supernate to a 2 × 16 cm column of DEAE-cellulose (DE-52; Whatman, Hillsboro, OR) equilibrated with suspension buffer from which the trypsin inhibitor was omitted. The column was washed with this buffer until the eluate was free of protein. After pooling the eluted fractions containing protein, we added trypsin inhibitor (1.5 g/L) and dialyzed the solution overnight, using dialysis membrane having a molecular-mass cutoff of about 12,000 Da, against buffer A: 10 mmol of Tris HCl (pH 7.2) and 1 mmol of benzamidine per liter. The dialysate was centrifuged and the supernate was applied to a second 1 × 20 cm
column of DEAE-cellulose that had been equilibrated with buffer A. The column was eluted with a linear gradient of NaCl (from 0 to 0.25 mol/L) in buffer A. Those 6-mL fractions containing procarboxypeptidase B were pooled, concentrated 10- to 30-fold by dialysis against a 500 g/L solution of polyethylene glycol (Aquadex III, 20 000 Da; Cal-Biochem, San Diego, CA) in buffer A, then dialyzed for 16 h against buffer A. The final concentration of procarboxypeptidase B in the dialysate was 2–5 g/L.

Criteria of purity. To evaluate the purity of the procarboxypeptidase B, we chromatographed 0.5-mL aliquots of the dialysates on TSK 3000SW and TSK 2000SW columns (Altex, Berkeley, CA) connected in series to obtain higher resolution, each 7.5 × 600 mm, equilibrated in 0.2 mol of sodium phosphate (pH 6.5) and 1 mmol of benzamidine per liter. We collected 1.0-mL fractions. The effluent was monitored at 285 nm. In addition, aliquots of the dialyzed pool containing 1 to 10 µg of protein were analyzed by polyacrylamide gel electrophoresis under denaturing conditions (22).

Molecular Mass Determination

We also subjected the procarboxypeptidase B dialysate to sodium dodecyl sulfate gel electrophoresis on a 12.5% polyacrylamide gel (20) and to sedimentation equilibrium centrifugation (Model E ultracentrifuge; Beckman Instruments, Palo Alto, CA) in potassium phosphate buffer (100 mM, pH 6.5) containing 0.2 mmol of benzamidine per liter.

Assay for Procarboxypeptidase B

To measure procarboxypeptidase B, we activated 10-µL samples containing procarboxypeptidase B by incubating them for 60 min at 37°C with 100 µL of bovine trypsin (Sigma), a 1 mg/mL solution in buffer containing 0.4 mol of Tris HCl (pH 8.3) and 4 mmol of CaCl₂ per liter. Carboxypeptidase B was assayed with hippuryl-L-arginine (Sigma) as substrate (21). One unit (U) of activity is defined as that required to hydrolyze 1 µmol of substrate per minute at 25°C (21). The difference in the amount of activity observed with and without activation by trypsin was used as a measure of procarboxypeptidase B activity. Protein concentrations were determined by the method of Bradford (22).

Production and Purification of Antibodies Specific for Carboxypeptidase B

Preparation of procarboxypeptidase B affinity gel. Procarboxypeptidase B, prepared as described above and shown by gel electrophoresis to be more than 95% pure and to contain about 0.6% active carboxypeptidase B, was used to prepare one affinity gel. An aliquot containing approximately 22 mg of protein, determined as described (22), was shaken for 3 h with 2 mL of Affi-gel 10 (Bio-Rad Laboratories, Richmond, CA) in 3 mL of pH 6.0 buffer containing 100 mmol of MES, [sodium 2-(N-morpholino)ethanesulfonate], 1 mmol of benzamidine, and 50 mmol of CaCl₂ per liter at room temperature, then left for 18 h at 4°C. The resulting affinity gel was washed with Tris HCl buffer (50 mmol/L, pH 7.6) containing 0.14 mol of NaCl per liter.

For another preparation of gel we used 20 mg of procarboxypeptidase B that was about 90% pure and 2 mL of Affi-Gel 10 in 3.6 mL of pH 7.5 buffer containing 100 mmol of HEPES [sodium 4-(2-hydroxyethyl)-1-piperazineethanesulfonate] and 50 mmol of CaCl₂ per liter. After 4 h at room temperature, the gel was incubated for 4 h at 4°C with 0.3 mL of 1 mol/L glycine, then washed with pH 7.3 buffer containing 10 mmol of Tris HCl and 1 mmol of benzamidine per liter.

Preparation of antiserum to carboxypeptidase B. We im-

munized a single rabbit with human native carboxypeptidase B, prepared as described previously (19), as a 1:2 emulsion with Freund complete adjuvant in a series of subcutaneous and intramuscular booster injections, 0.1 mg each, as previously described (19, 23). The antibody titer of the rabbit antiserum, defined as the dilution of antiserum required to precipitate 50% of a fixed amount of labeled tracer in a double-antibody assay, was determined with 125I-labeled carboxypeptidase B as described (23). We also titrated the antiserum with 125I-labeled procarboxypeptidase B to determine the relative cross reactivity between procarboxypeptidase B and carboxypeptidase B. Both carboxypeptidase B and procarboxypeptidase B were labeled with 125I by use of the Chloramine T method (23).

Treatment of anti-carboxypeptidase B antibodies with procarboxypeptidase B affinity gel. To estimate the affinity gel's capacity we added, in sequence, a total of ten 0.1-mL aliquots of procarboxypeptidase B affinity gel to 1 mL of rabbit anti-carboxypeptidase B serum. After each such addition, the mixture was shaken for 20 min at room temperature, then centrifuged (1100 × g, 2 min), and a 50-µL sample of the supernate was removed. We determined the titer of each supernate to carboxypeptidase B and procarboxypeptidase B as described above.

In a second experiment we incubated 0.75 mL of gel with 1.0 mL of antiserum for 1 h at room temperature, then filtered the supernate liquid through a 0.45-μm filter (Millex-HA; Millipore Corp., Bedford, MA).

We used two separate batches of antibody, each prepared from one of the affinity gels described above, in single-tube assays of active carboxypeptidase B.

Immunooassays

Radioimmunoassays for carboxypeptidase B. We radioimmunoassayed carboxypeptidase B as described elsewhere (23), using either the initial polyclonal antiserum or the affinity-purified specific antibodies. We diluted the original antiserum 1 200 000-fold and the specific antibodies 20 000-fold in the respective assays. We set up standard curves as described previously (23) with concentrations of standard carboxypeptidase B ranging from 0.1 to 3 μg/L for the untreated initial antiserum and 1.5 to 45 μg/L for gel-treated antiserum. Statistical analysis was performed as previously described (29).

Immunoenzymometric assay. We developed a "sandwich" type assay, which included the following steps: (a) guinea pig anti-carboxypeptidase B IgG was bound to microtiter plates by overnight incubation of a 1 µg/mL solution, 200 µL per well, at 37°C; (b) serum antigen or standard carboxypeptidase B was allowed to bind to the plate-bound IgG by incubation of 200 µL (per well) of an appropriate dilution of serum (two- to 10-fold) or standard (0.1 to 5 ng) for 1 h at 37°C; (c) incubation of a 500-fold dilution of purified rabbit anti-carboxypeptidase B IgG, 200 µL per well, for 1 h at 37°C; (d) incubation of an 800-fold dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG (Miles Laboratories, Elkhart, IN), 200 µL per well, for 1 h at 37°C; and (e) quantification of alkaline phosphatase activity with use of 200 µL of a 1 mg/mL solution of p-nitrophenyl phosphate in buffer containing 1 mol of diethanolamine (pH 9.8) and 1 mmol of MgCl₂ per liter. Assay mixtures were incubated for 30 min at 37°C and the absorbance at 410 nm measured with an automatic plate reader (Flow Labs., Rockville, MD).

Amylase Assay

Amylase activity concentrations in serum were determined in an ace (Du Pont) discrete analyzer.
Size Fractionation of Human Serum Immunoreactive Carboxypeptidase B

We chromatographed 50- to 500-μL serum samples on a double column of TSK-3000SW and TSK-2000SW, connected in series, in pH 6.8 buffer containing 0.2 mol of sodium phosphate and 1 mmol of benzamidine per liter. We collected 0.25-mL fractions into tubes containing 0.55 mL of pH 7.6 buffer (50 mM of Tris HCl, 0.14 mol of NaCl, 4 g of bovine serum albumin, and 0.12 g of rabbit IgG per liter) and radioimmunoassayed each fraction for carboxypeptidase B, using untreated or gel-treated antibodies.

For some serum samples, we assayed effluent fractions for carboxypeptidase B and procarboxypeptidase B by immunoenzymometric assay. For either immunooassay, we calculated the amount of carboxypeptidase B in a serum sample by summing the concentrations of carboxypeptidase B in the peak corresponding in elution position to active enzyme, and multiplying by factors for the fraction size and the amount of sample applied to the column.

Patients

We analyzed a total of 10 samples from five patients with acute pancreatitis. The first four samples listed in Table 1 were daily serum samples from a 12.6-year-old girl who developed acute pancreatitis after being admitted to the hospital with a diagnosis of Raye's syndrome; she died the day the final sample was taken. Sample 5 was from a patient with gallstone-induced edematous pancreatitis, who recovered. Sample 6 was drawn on the second hospital day from a patient with acute hemorrhagic pancreatitis, who died on the fifth hospital day. Samples 7–9 were drawn on hospital days 3, 4, and 5 from a 30-year-old man with acute alcoholic hemorrhagic pancreatitis, who recovered. Data from an additional sample, drawn on the first hospital day from a 44-year-old man with acute alcoholic edematous pancreatitis, who recovered, are included below in Figure 5.

Protein Standards

Human pancreatic carboxypeptidase A (EC 3.4.17.1) was previously purified to homogeneity in our laboratory. Human pancreatic carboxypeptidase N (EC 3.4.17.3) was kindly provided by Dr. Thomas Plummer, State of N.Y. Dept. of Health, Albany, NY.

Table 1. Active Carboxypeptidase B in Serum As Determined by Direct Radioimmunoassay and by Size Fractionation of Total Immunoreactive Carboxypeptidase B

<table>
<thead>
<tr>
<th>Sample</th>
<th>Single-tube RIA</th>
<th>Column fractionation*</th>
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<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>_b</td>
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<td>2</td>
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<td>3</td>
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<td>32</td>
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<tr>
<td>8</td>
<td>11</td>
<td>74</td>
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</tbody>
</table>

*Serum samples from patients with acute pancreatitis were fractionated on a TSK 3000/2000 liquid-chromatographic column and each fraction was assayed for immunoreactive carboxypeptidase B with untreated antisera as shown in Fig. 4. Values for the fractions corresponding to the elution position of purified active carboxypeptidase B were summed to yield the concentration of active carboxypeptidase in the sample. Column fractions from samples 1 through 4 were assayed for carboxypeptidase B with an immunoenzymometric assay, those from samples 5 through 9 with a radioimmunoassay.

Statistical Analysis

Radioimmunoassay data were linearized by using a logit transformation (23). Pearson's correlation analysis was used to characterize the relationships between concentrations of carboxypeptidase B and trypsin-α1-protease inhibitor complex or amylase activity in serum as well as the accuracy of the single-tube assay as compared with the column fractionation method for measurement of carboxypeptidase B.

Results

Purification and Characterization of Procarboxypeptidase B

Success in purifying the zymogen depended on use of fresh normal pancreatic tissue containing little active trypsin, <1% relative to trypsinogen. When we used one such powder, we obtained a symmetrical peak for procarboxypeptidase B from the second DEAE-cellulose column at a NaCl concentration of about 0.12 mol/L. This material also was eluted as a single symmetrical peak from a TSK-Sphero gel column. The fractions corresponding to the peak contained material that appeared to be >96% pure by gel electrophoresis (Figure 1, lane A), which we judged suitable for preparation of affinity gels. On incubation with trypsin the proenzyme was completely converted to a protein that migrated with the electrophoretic mobility of carboxypeptidase B (21) and low-molecular-mass fragments (Figure 1, lane D). The trypsin-activated material had a specific activity of 72.7 kU/g. As shown in Table 2, the yield of procarboxypeptidase B was 61% and the specific activity was increased by about 11-fold.

A second preparation of procarboxypeptidase B from a different acetone powder yielded zymogen that was about 85% pure with respect to protein, containing 0.2% active carboxypeptidase B (Figure 1, lane B).

We estimated the relative molecular mass of human

![Fig. 1. Migration of human pancreatic procarboxypeptidase B during sodium dodecyl sulfate/polyacrylamide gel electrophoresis](image-url)

A, procarboxypeptidase B used for affinity-gel preparation by Method I and for cross-reactivity experiments; B, procarboxypeptidase B used for affinity-gel preparation by Method II; C, control lane, no protein added; D, migration of human pancreatic carboxypeptidase B after activation with trypsin; E, porcine pancreatic carboxypeptidase B, 1 μg; F, molecular mass standards, marked in kilodaltons. Application point is at top.
procarboxypeptidase B to be about 46 000, as judged from its mobility during gel electrophoresis. The relative molecular mass of human procarboxypeptidase B was determined to be between 44 000 and 48 000 by ultracentrifugation, assuming a partial specific volume of 0.73 to 0.75 cm³ g⁻¹. Human procarboxypeptidase B and carboxypeptidase B both interact with the TSK SW-type steric-exclusion resins. In the absence of a buffer with high ionic strength they emerge in poor yield and only after many column volumes. At high ionic strengths (e.g., 0.2 mol of sodium phosphate per liter, pH 6.8) procarboxypeptidase B (46 000 Da) is eluted after ovalbumin (40 000 Da), and carboxypeptidase B (35 000 Da) after chymotrypsin (25 000 Da) and myoglobin (17 000 Da).

Affinity Purification of Specific Antiserum

As shown in Figure 2, the initial polyclonal antisera to carboxypeptidase B had an extremely high titer (≈ 2 x 10⁶) for binding to either active carboxypeptidase B or the zymogen. As also shown in Figure 2, when we incubated antiserum to carboxypeptidase B sequentially with aliquots of procarboxypeptidase B gel, there was a progressive loss of antibodies to both carboxypeptidase B (Figure 2, part A) and procarboxypeptidase B (part B). Antibodies that react with procarboxypeptidase B were removed and to a greater extent than antibodies to carboxypeptidase B. These data show that, after about four additions of gel, antibodies to carboxypeptidase B began to be removed more slowly, while that of antibodies to procarboxypeptidase B continued at the initial rate. After about six additions, removal of antibodies to procarboxypeptidase B was dramatically decreased, and appeared to occur at approximately the same rate as that of antibodies to carboxypeptidase B.

The resulting titration curves for the original untreated antiserum and treated antibodies are shown in Figure 3.

Guided by these gel-aliquot experiments, we performed a single batch adsorption with gel on another aliquot of antiserum to obtain a large amount of specific antibodies to carboxypeptidase B. The resulting titration curves for the original untreated antiserum and treated antibodies are shown in Figure 3.

Specificity of absorbed antiserum. When we used the gel-treated antibodies in a radioimmunoassay for carboxypeptidase B, the standard curve was linear and paralleled that obtained with untreated antibodies (results not shown). The mean slope for five standard curves, prepared during three months, was 0.965 (SD 0.065), while the mean X₀ value (defined by Rodbard (24) as the dose of antigen required to achieve a B/B₀ of 0.5) was 3.5 (SD 1.1) ng of carboxypeptidase B. The sensitivity was approximately a fifth as great as that achieved with the untreated antibodies (minimum detectable dose = 0.5 vs 0.1 µg/L) as determined according to previously described methods (23). Using gel-treated antibodies, a purified sample of procarboxypeptidase B that contained about 5 mg of carboxypeptidase B per gram, as estimated from enzyme activity, reacted with an efficiency of approximately 0.2% in the radioimmunoassay. Neither human pancreatic carboxypeptidase A nor human pancreat-
We fractionated serum samples from patients with acute pancreatitis on the TSK 3000/20000 columns, to test the specificity of the affinity gel treated antibodies for active carboxypeptidase B. Figure 4 shows a representative profile of serum immunoreactive carboxypeptidase B from a patient with acute pancreatitis, assayed in separate radioimmunoassays with both gel-treated and untreated anti-carboxypeptidase B antibodies. The original untreated antibodies detected two major peaks of immunoreactive material—procarboxypeptidase B and active carboxypeptidase B. In contrast, the affinity-gel-treated antibodies detected the active enzyme, but not thezymogen. In addition, there appears to be a small amount of immunoreactive material eluting before procarboxypeptidase B, as demonstrated by the peak detected with the treated antibodies and the shoulder detected with the untreated antibodies at about 41 min. When authentic carboxypeptidase B was added to normal serum before chromatography on this column, small and variable amounts of immunoreactive material were eluted at this position. When normal sera were size-fractionated, a single peak of immunoreactive material (procarboxypeptidase B) was detected with the untreated antisera, and essentially no immunoreactive material was detected with the gel-treated antibodies (results not shown). In accordance with these results, single-tube assays of samples of normal serum by use of the treated antibodies showed no detectable active carboxypeptidase B, while approximately 5 to 10 ng of procarboxypeptidase B per liter was detected by a single-tube assay with use of untreated antisera.

In contrast to data on normal sera, normal plasma from the same individuals appeared to contain high concentrations of immunoreactive active carboxypeptidase B when assayed with treated antibodies. However, values obtained with three different quantities of plasma were extremely non-parallel to the standard curve. In addition, these values were substantially greater than the concentrations of immunoreactive carboxypeptidase B we detected by using untreated antisera.

Accuracy of the Single-Tube Method

We investigated the specificity and accuracy of the single-tube assay for measurement of concentrations of active carboxypeptidase B in serum by comparison with values for active carboxypeptidase B determined by gel filtration of serum and immunoassay of the resulting effluent fractions. In some of the column-fractionation experiments we used radioimmunoassay to measure the concentration of carboxypeptidase B; in others, we used an immunoenzymometric method developed for use in other experiments. The effluent profiles for carboxypeptidase B in serum were similar to that shown in Figure 4 (untreated antibodies curve). As shown in Table 2, except for the last-listed sample, there was excellent agreement between results by the single-tube method and the column-fractionation method (for all samples, r = 0.996), especially given the relatively high error associated with summation of data over a chromatographic peak.

Correlation of Active Carboxypeptidase B in Serum with Inhibitor-Bound Active Trypsin

Figure 5 shows the correlation between active carboxypeptidase B as determined by the single-tube assay and the concentration of α1-protease-inhibitor-bound cationic trypsin in sera from patients with acute pancreatitis. The amount of active carboxypeptidase B in serum correlates well (r = 0.83, p < 0.03) with the values for active trypsin detected in complex with the inhibitor in serum. In contrast, neither active carboxypeptidase B nor inhibitor-bound trypsin is correlated with serum amylase (r = −0.135 and r = −0.09, respectively).

Discussion

The proteolytic enzymes are synthesized and normally are stored in the zymogen granules as proenzymes. Either zymogens or active enzymes might be released during episodes of acute pancreatitis. We have previously hypothesized that an assay measuring the amount of active enzyme in a blood sample (as contrasted to the amount of zymogen) might be a useful diagnostic tool to assess the degree of pancreatic zymogen activation and perhaps predict morbidity in acute pancreatitis (13, 15). Borgstrom and Lason
recently found that the concentrations in serum of trypsin-α-protease inhibitor complex, formed by release of active trypsin from the pancreas, were correlated with morbidity in patients with acute pancreatitis. In contrast, neither total immunoreactive trypsin or amylase was a good measure of morbidity in these patients. We have also reported a correlation between concentrations of this complex in serum and mortality in an experimental model of pancreatitis in rats (16). Both of these studies required size fractionation of serum followed by radioimmunoassay of the resulting effluent fractions to distinguish inhibitor-bound trypsin from trypsinogen.

We desired a simple, single-tube immunoassay for the concentration in serum of the active form of a pancreatic enzyme normally synthesized as a zymogen. As noted above, amylase and lipase are not synthesized as zymogens (12) and thus measurement of these enzymes cannot yield information on the degree of zymogen activation. In addition, enzymatic measurement of pancreatic enzymes in blood is plagued by both a lack of specificity (26) and the fact that trypsin (5), chymotrypsin (6), and elastase (9) all react with inhibitors in serum to form castronically inactive complexes. The initial finding in the current study—that there was a relatively large difference in relative molecular mass between human pancreatic carboxypeptidase B (M , 35,000) and its zymogen (M , 46,000)—suggested that, during inactivation, release of the large activation peptide may expose previously buried antigenic determinants. We hypothesized that antibodies to an epitope of a polyclonal antisera raised to carboxypeptidase B that would be specific for this "buried" region, and that would serve as the basis for a specific immunoassay for active carboxypeptidase B.

To obtain antibodies specific for active carboxypeptidase B we chose to remove the antibodies from a polyclonal antisera that reacts with the zymogen by using an affinity gel of procarboxypeptidase B. Although this method does not require pure procarboxypeptidase B it does demand that the material be essentially free of active carboxypeptidase B. Despite the extensive use of protease inhibitors and pH conditions designed to minimize trypsin activity, it was difficult to obtain procarboxypeptidase B absolutely free of active enzyme and to maintain this condition throughout the zymogen-gel coupling and affinity-adsorption steps. The presence of trace amounts of active enzyme on the procarboxypeptidase affinity gel undoubtedly contributes to the low yield of specific antibodies. The data shown in Figure 2 on affinity-gel treatment of antisera demonstrate that, initially, antibodies directed toward epitopes common to both carboxypeptidase B and procarboxypeptidase B, which account for the majority of the population, were adsorbed by the affinity gel. The much slower loss of antibodies to carboxypeptidase B after four additions of gel demonstrates that there is a small population of antibodies directed to epitopes specific for carboxypeptidase B, which is not removed efficiently by the immobilized procarboxypeptidase B. Extrapolation of the curve to zero-additions of procarboxypeptidase B gel suggests that about 10% of the original population of antibodies was specific for carboxypeptidase B. We assume that these antibodies continue to be removed at a low rate by further additions of procarboxypeptidase B affinity gel because of traces (between 0.2 and 1%) of carboxypeptidase B on the gel. Theoretically, subsequent additions of affinity gel should have continued to remove antibodies to procarboxypeptidase B at the initial rate. The break in the procarboxypeptidase B curve probably does not represent a differential removal of antibodies against the zymogen; rather, it represents apparent antibody reactivity due to the presence of a contaminating concentration of 125I-labeled carboxypeptidase B in the sample of 125I-labeled procarboxypeptidase B used to titrate the anti-carboxypeptidase B antibodies. The low yield of specific antibody should be overcome by use of a monoclonal antibody for active carboxypeptidase B, the production of which is now in progress in our laboratory. Nevertheless, the present study demonstrates the feasibility of obtaining an antibody population specific for active carboxypeptidase B.

The specificity of the absorbed antibodies was demonstrated both by the low cross-reactivity (0.2%) with procarboxypeptidase B standard in vitro and by the lack of detection of procarboxypeptidase B in fractionated sera (Figure 4). However, we have noticed when we fractionate sera from pancreatitis patients and assay for carboxypeptidase B with either the specific antibodies or the original polyclonal antisera, a small peak corresponding to apparent carboxypeptidase B is detected eluting in front of the position of procarboxypeptidase B (Figure 4). This may represent either carboxypeptidase B dimer or perhaps a small amount of the material that interferes in the assay of plasma samples. The fact that there is no cross-reactivity between either human pancreatic carboxypeptidase A or human serum carboxypeptidase N (25) in the assay with either treated or untreated antisera indicates that the peak appearing on liquid chromatography is not ascribable to these proteins. To demonstrate that the single-tube assay accurately measures the concentration of active carboxypeptidase B in serum samples, we subjected samples to size fractionation, then measured the amount of carboxypeptidase B in the peak corresponding to active enzyme, either by radioimmunoassay or immunoenzymometric assay. As shown for a representative serum sample in Figure 4, two peaks of immunoreactive material, corresponding to procarboxypeptidase B and carboxypeptidase B, were detected in sera from patients with acute pancreatitis on using polyclonal antisera. These peaks reflect the release of both zymogen and active enzyme into the bloodstream during the disease process. Comparison of the data obtained by the single-tube procedure and the column-fractionation method (Table 1) shows that the former successfully measures the amount of active pancreatic carboxypeptidase B in sera of patients with acute pancreatitis.

As discussed above, we (13–16) and others have presented evidence for a possible correlation between the amount of inhibitor-bound serum trypsin and the pathological signs as well as the morbidity associated with acute pancreatitis. As shown in Figure 5, our preliminary results suggest that active carboxypeptidase B concentrations in serum parallel the amounts of inhibitor-bound trypsin. Furthermore, there is no correlation between the concentrations of either active carboxypeptidase B or active trypsin bound to α-protease inhibitor and serum amylase values. We interpret this as reflecting a substantial release of amylase and zymogens in some cases of mild acute pancreatitis, while in cases where zymogen activation is significant, serum amylase concentrations may not be increased further. This finding emphasizes our view that measurement of active enzymes in serum may be of greater predictive value or, at the least, might indicate more accurately the degree of pancreatic zymogen activation than does the currently standard amylase test. Future studies will be directed toward the use of this assay to confirm the correlation between carboxypeptidase B and trypsin and to assess the utility of assay of active carboxypeptidase B in serum for diagnosis and prognosis of acute pancreatitis.

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