Automated Assay for $\alpha_1$-Antitrypsin with N-$\alpha$-Benzoyl-DL-arginine-$p$-nitroanilide as Trypsin Substrate and Standardized with $p$-Nitrophenyl-$p'$-guanidinobenzoate as Titrant for Trypsin Active Sites

John H. Eckfeldt,¹ R. Todd Light, and Catherine Lelendecker-Foster

$\alpha_1$-Antitrypsin is the most abundant of several serum protease inhibitors. Its deficiency is associated with an increased incidence of emphysema in adults, jaundice in newborns, and childhood cirrhosis. We describe an automated functional assay for the Instrumentation Laboratory’s Multistat III Microcentrifugal Analyzer with N-$\alpha$-benzoyl-DL-arginine-$p$-nitroanilide as trypsin substrate. The assay is standardized in terms of moles of trypsin active sites inhibited per liter of serum, by use of a chromogenic titrant for trypsin active sites, $p$-nitrophenyl-$p'$-guanidinobenzoate. The method is rapid, precise, and independent of trypsin supplier, and results correlate well with those by a manual chromogenic and a nephelometric assay.

Additional Keyphrases: screening · centrifugal analyzer · heritable disorders

Several electrophoretically distinct plasma glycoproteins, including $\alpha_1$-antitrypsin, $\alpha_2$-macroglobulin, $\alpha_2$-antiplasmin, and $\alpha_1$-antichymotrypsin, are capable of inhibiting various proteolytic enzymes containing active-site serine residues. The most thoroughly studied of these serine protease inhibitors is $\alpha_1$-antitrypsin, which has a molecular mass of approximately 54 000 daltons, is about 12% carbohydrate (contained on four separate side chains), accounts for about 90% of the $\alpha_1$-band in cellulose acetate electrophoresis, and contributes about 90% of the antiproteolytic activity of serum (1–6). It is coded for by a pair of codominant, completely penetrant, autosomal alleles at the Pi (protease inhibitor) locus that exhibit a high degree of polymorphism, having more than 20 different alleles reported so far (4–6). Most $\alpha_1$-antitrypsin deficiency states are caused by the Z and S alleles, which result in decreased amounts of $\alpha_1$-antitrypsin protein in serum. A patient who is a homozygote for one of these alleles, or is a heterozygote combining the two of them, is at increased risk for developing pulmonary or hepatic disease (3–6).

Screening for $\alpha_1$-antitrypsin deficiency has generally been done by quantitating the $\alpha_1$-band in cellulose acetate electrophoresis or by quantitating $\alpha_1$-antitrypsin by techniques such as radial immunodiffusion, rocket electrophoresis, or nephelometry. Patients found deficient by such techniques are usually then tested for $\alpha_1$-antitrypsin phenotype by crossed immunoelectrophoresis (7, 8) or by isoelectric focusing (9, 10), because such patients cannot be reliably separated from heterozygotes having the normal M allele by quantitation of $\alpha_1$-antitrypsin alone.

Measurement of trypsin-inhibitory capacity has not found widespread use because most assays are manual and laborious (11–13), though a few automated procedures have been described (14, 15). $\alpha_1$-Antitrypsin variants lacking anti-proteolytic properties, while theoretically possible, have not been discovered; one might speculate that this is because most large-scale screening studies (16–19) have involved techniques that detect only patients lacking the $\alpha_1$-antitrypsin protein in their serum.

Another problem with current assays for trypsin-inhibitory capacity has been lack of absolute standardization, making meaningful interlaboratory comparisons difficult (6). The amount of trypsin (EC 3.4.21.4) inhibited by a given volume of serum has been variably expressed either as mass or as units of trypsin activity inhibited. However, commercially available crystallized trypsins are not 100% enzymically active; each contains variable proportions of trypsinogen, enzymically active $\alpha$- and $\beta$-trypsins, and inactive fragments (20). Normally, 40 to 70% of the trypsin, determined by weight or by absorbance at 280 nm, is active, when $p$-nitrophenyl-$p'$-guanidinobenzoate (NPGB) is used as an active-site titrant (21). Because $\alpha_1$-antitrypsin interacts only with enzymically active trypsin, the mass of trypsin inhibited per volume of serum depends on the purity of the trypsin used.

Expression of trypsin-inhibitory capacity as trypsin enzymic units inhibited per volume of serum also has drawbacks. The substrate most commonly used, N-$\alpha$-benzoyl-DL-arginine-$p$-nitroanilide (BAPNA), has a $K_m$ of 0.8 mmol/L but a solubility of only about 0.3 mmol/L at room temperature in the pH 8.3 Tris buffers ordinarily used. Thus, the trypsin is operating at nonsaturating substrate concentrations, and the units of trypsin activity inhibited will depend on the exact substrate concentrations used in the assay. Expression of trypsin-inhibitory capacity as units of trypsin inhibited has another theoretical problem in that $\beta$-trypsin has been reported to hydrolyze BAPNA 50% more rapidly than does $\alpha$-trypsin (22). If this is so, then the more $\beta$-trypsin in a commercial trypsin preparation, the more units of trypsin will be inhibited per unit volume of serum.

In this paper we describe a rapid, precise automated assay for serum trypsin-inhibitory capacity. The assay is standardized in terms of moles of trypsin active sites inhibited per liter of serum; unlike most previous assays, the results are independent of trypsin supplier. This assay should facilitate measurement of trypsin-inhibitory capacity of serum and improve interlaboratory comparability.

Materials and Methods

We used serum specimens and noted no change in trypsin-inhibitory capacity in serum stored refrigerated for five days, frozen for one month at $-20 {^\circ}C$, or frozen for one year at $-70 {^\circ}C$.

Apparatus

We used a Multistat III Microcentrifugal Analyzer (Instrumentation Laboratory, Lexington, MA 02193), the principle, design, and operation of which have been described elsewhere (23). For comparison experiments we used the manual chromogenic assay of Eriksson (17) and a commercial

¹ Direct reprint requests to this author, at Laboratory Service 113, V A Medical Center, Minneapolis, MN 55417.

Received Dec 31, 1981; accepted Feb 2, 1982.
Reagents

BAPNA, NPGB, and benzamidine were obtained from Sigma Chemical Co., St. Louis, MO 63178. Twice-crystallized bovine pancreatic trypsin was obtained from Sigma (no. T9255) for routine experiments. For comparison experiments we used crystallized bovine trypsin from several sources (no. 109819, Boehringer-Mannheim, Indianapolis, IN 46250; no. 6510, Calbiochem-Behring, La Jolla, CA 92037; no. TRL 50S, Worthington Diagnostics, Freehold, NJ 07728). Dimethylsulfoxide (methyl sulfoxide) and N,N-dimethylformamide were obtained from Eastman Organic Chemicals, Rochester, NY 14650; SP-Sephadex from Pharmacia Fine Chemicals, Piscataway, NJ 08854; and B-15 Minicon concentrating blocks from Amicon Corp., Lexington, MA 02173. All other chemicals were AR grade.

Stock NPGB: 67.4 g of NPGB per 10 mL of N,N-dimethylformamide; this mixture is stable for at least four months at 4 °C.

Tris buffer: per liter, 40 mmol of calcium chloride and 200 mmol of Tris hydrochloride, pH 8.43 at 25 °C; stable at least six months at 4 °C.

Stock trypsin solution: 300 mg of trypsin per 10 mL of 1.0 mmol/L hydrochloric acid stored frozen as 100-µL aliquots; stable at least six months at −20 °C.

Working trypsin solution: Combine 3.9 mL of Tris buffer with 100 µL of stock trypsin solution; stable 2 h at 4 °C.

Stock BAPNA solution: 218 mg of BAPNA in 5 mL of dimethyl sulfoxide; stable at least three months at 4 °C.

Working BAPNA solution: Combine 25 µL of stock BAPNA solution with 12.5 mL of Tris buffer at 37 °C; stable 2 h at 37 °C.

Stock dichromate solution: 10 g of potassium dichromate per liter of 50 mmol/L sulfuric acid; stable two years.

Dichromate, 1.67 g/L: Dilute stock dichromate sixfold with 50 mmol/L sulfuric acid.

Dichromate, 80 mg/mL: Using class-A volumetric glassware, combine 2 mL of stock dichromate solution with 200 mL of 50 mmol/L sulfuric acid and dilute to 250 mL with distilled water.

Controls

One control was prepared from pooled human serum. A low control was prepared by diluting the first control fivefold with bovine serum albumin, 50 g/L. Both controls were aliquotted and stored at −70 °C.

Procedures

Multistat III loader calibration: Using the loader settings in Table 1 and 50 mmol/L sulfuric acid in the reagent boat, load a rotor as follows: cuvette 1 to 4, water as sample and second reagent; cuvettes 5 to 8, water as sample and 1.67 g/L dichromate as second reagent; cuvettes 9 to 12, 10 g/L stock dichromate as sample and water as second reagent; cuvettes 13 to 16, hand load with 200 µL of 80 mg/L dichromate. Measure the absorbances at 340 nm (General Absorbance Program no. 24, Substrate II tape) in the analyzer and calculate the mean absorbances for wells 1 to 4 (A0), for wells 5 to 8 (A1), for wells 9 to 12 (A2), and for wells 13 to 16 (A3). Calculate the fractional delivery for serum (f1,s) and for trypsin (f1,t) as follows:

\[ f_{1,s} = (A_1 - A_0)/(A_3 - A_0) \]
\[ f_{1,t} = (A_2 - A_0)/(A_3 - A_0) \]

For our loader, f1,s was 1.02 and f1,t was 0.88 when using the loader settings in Table 1.

<table>
<thead>
<tr>
<th>Sample syringe:*</th>
<th>Sample vol. 14% (12 µL of working trypsin) b</th>
<th>Sample vol 2% (2 µL of water or serum)</th>
<th>Total vol 25% (25 µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent syringe:*</td>
<td>Reagent vol. 80% (200 µL of working BAPNA)</td>
<td>Reagent vol. 90% (225 µL)</td>
<td></td>
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</table>

* The settings on the Multistat III Loader are expressed as percentages of the 100-µL sample syringe and the 250-µL reagent syringes. b The second reagent volume actually delivered is the second reagent volume setting minus the sample volume. The difference between this second reagent volume setting or the reagent volume setting and the total volume is the amount of distilled water that washes the sample and (or) reagent into the rotor cuvette. c Distilled water is delivered into cuvette 1 when the Reagent/Diluent switch is set to Diluent.

Isolation of α- and β-trypsins: These two active trypsin isoforms were separated chromatographically at 4 °C as described by Schroeder and Shaw (22), with slight modifications. Equilibrate a 1 × 60 cm column of SP-Sephadex with 0.11 mol/L Tris hydrochloride, pH 7.1 (at 25 °C), containing 20 mmol of calcium chloride and 1 mmol of benzamidine per liter. Apply 60 mg of twice-crystallized bovine trypsin dissolved in 1 mL of the equilibration buffer and elute the column with the same buffer at 2.5 mL/h, collecting 1-1 mL fractions. After measuring the absorbances of the fractions at 280 nm, combine the fractions containing α-trypsin and combine the fractions containing β-trypsin (the second and third peaks, respectively), dialyze each extensively against 1 mmol/L hydrochloric acid, and concentrate with an Amicon B15 concentrating block.

Measurement of protein concentration of trypsin solutions: Measure the absorbance at 280 nm and calculate the molar concentration from the absorptivity of trypsin at 280 nm, 1.54 g·cm⁻¹·L⁻¹ (20), and its molecular mass, 24 000 daltons (20).

Measurement of active-site concentration of trypsin solutions: Measure the absorbance at 402 nm of 1 mL of working solution of trypsin. Add 10 µL of stock NPGB, noting the time, and record the absorbance at 402 nm for approximately 1 min. Extrapolate the gradually increasing absorbance back to the time of addition of the NPGB and calculate the "burst" of absorbance at 402 nm for the working trypsin solution (b, approximately 0.32 A). This "burst" results from rapid stoichiometric release of p-nitrophenol as trypsin's active-site serines cleave the NPGB. Do a similar experiment with Tris buffer without trypsin and calculate the "blank burst" of absorbance at 402 nm for the Tris buffer alone (b0, approximately 0.02 A). Calculate the active-site concentration of the working trypsin solution (a) as shown below (21):

\[ a, \text{mol/L} = (b - b_0)/18300 \]

where 18 300 is the molar absorptivity (mol·cm⁻¹·L⁻¹) for p-nitrophenol in the Tris buffer at 402 nm, as we determined for p-nitrophenol recrystallized from benzene.

Measurement of trypsin-inhibitory capacity of serum:
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Figure 1. Lineweaver-Burk plot for BAPNA for trypsin alone (■) and with 2 μL of pooled serum added per cuvette (○)
The cuvette concentrations of trypsin, Tris, and calcium were 48 mg/L, 160 mmol/L, and 32 mmol/L, respectively.

Load the patients' and control serum specimens into the Multistat III rotor, using the loader settings and configuration as shown in Table 1. Analyzer settings are as follows: tape, enzyme program, 23 (general enzyme); reference cuvette, 1; delay interval, 7 s; data time interval, 3 s; number of data points, 11; filter code, 3 (405 nm); start mode, 0 (incubate to temperature before mixing); absorbance maximum, 2; temperature, 2 (37 °C); factor, 10 000. Calculate the fractional inhibition of the trypsin activity, fi, from the mean reaction velocities with water in the sample cups (vw) and with serum in the sample cup (vs):

\[ f_i = (v_w - v_s)/v_w \]

Then calculate the moles of trypsin active sites inhibited per liter of serum from the active-site concentration (a), the volume of working trypsin added to each cuvette (12 × 10^{-6} L), the volume of serum added to each cuvette (2 × 10^{-6} L), the Multistat III Loader fractional delivery corrections for serum (fda) and for working trypsin (fda), and the fractional inhibition of trypsin activity (fi) as follows:

moles of trypsin active site inhibited per liter of serum

\[ = (a \cdot 12 \times 10^{-6} \cdot f_{da} \cdot f_i)/(2 \times 10^{-6} \cdot f_{da}) \]

Method comparisons: The nephelometric α1-antitrypsin and the manual trypsin-inhibitory capacity were determined as described by Beckman Instruments (24) and Eriksson (11), respectively.

Results and Discussion

Figure 1 shows the Lineweaver–Burk plot for trypsin with BAPNA as the substrate with and without addition of serum. The inhibition pattern is “pure noncompetitive” indicating that the serum inhibitor has no influence on the K̄m of BAPNA for trypsin (25). This result is consistent with the proposed irreversible, covalent inactivation of trypsin by α1-antitrypsin (26).

Optimally, enzyme assays should be conducted at substrate concentrations several times higher than the substrate's K̄m for the enzyme. However, the limited solubility of BAPNA makes it necessary to conduct the assay near or below the K̄m of 0.8 mmol/L. Furthermore, we found that initial velocities were more stable at BAPNA concentrations somewhat below the solubility limit of the substrate. We presume this is due to the crystallization of the BAPNA in the working BAPNA, which we observed repeatedly when concentrations above about 0.3 mmol/L were used. We selected a final concentration of 0.20 mmol/L for the working solution of BAPNA and observed no decrease in velocity from product inhibition or substrate depletion during the initial 40 s of the reaction, in which only about 5% of the available substrate is consumed.

In the method presented, an absolute measure of the trypsin concentration is provided by the active-site titration rather than from mass of trypsin or from the trypsin activity as discussed previously. The absolute reaction rates are unimpor-

Fig. 2. Initial velocity vs trypsin concentration, for trypsin alone (○) and for trypsin plus 2 μL of pooled serum per cuvette (■)
The cuvette concentrations of Tris, calcium, and BAPNA were 160, 32, and 0.16 mmol/L, respectively. The trypsin concentrations (x-axis) are final concentrations in the cuvette.

Fig. 3. Initial velocity vs volume of serum added
The cuvette concentration of Tris, calcium, and BAPNA were 160, 32, 0.16 mmol/L, respectively, and the trypsin concentration in the cuvette was 36 mg/L.
Table 2. Comparison of the Antitryptic Activity of a Pooled Serum on Trypsin from Different Sources

<table>
<thead>
<tr>
<th>Trypsin source (and % activity)</th>
<th>Mean ( μmol of trypsin active-sites per liter of serum)</th>
<th>Inhibited</th>
<th>Arbitrary units of trypsin activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sigma (64%)</td>
<td>44.3 (2.0)</td>
<td>69.2 (5.5)</td>
<td>100 (4)</td>
</tr>
<tr>
<td>Calbiochem-Behring (52%)</td>
<td>44.4 (0.3)</td>
<td>85.3 (0.5)</td>
<td>106 (1)</td>
</tr>
<tr>
<td>Boehringer-Mannheim (49%)</td>
<td>44.1 (0.2)</td>
<td>90.5 (0.5)</td>
<td>111 (1)</td>
</tr>
<tr>
<td>Worthington (70%)</td>
<td>45.1 (1.4)</td>
<td>64.9 (2.0)</td>
<td>93 (3)</td>
</tr>
<tr>
<td>α-Trypsin (62%)</td>
<td>44.3 (1.5)</td>
<td>71.4 (2.2)</td>
<td>97 (3)</td>
</tr>
<tr>
<td>β-Trypsin (84%)</td>
<td>42.7 (0.3)</td>
<td>52.1 (0.5)</td>
<td>97 (1)</td>
</tr>
</tbody>
</table>

* % activity is based on simultaneous measurement of the active-site concentration and the protein concentration of the working trypsin solution as described in the text. b Calculated as described in the text. c Calculated by substituting the protein concentration of the working trypsin solution, as determined by absorbance at 280 nm for the working trypsin active-site concentration (a); see text.

Comparisons of serum's antitryptic capacity determined on the basis of trypsin active-site inhibition, trypsin protein inhibition, and trypsin activity inhibition are shown in Table 2. Note the marked variability in the percent activity of trypsin from various suppliers, as Chase and Shaw previously observed (21). As expected, the micromoles of trypsin protein inhibited per liter of serum varies inversely with the trypsin purity, since the α1-antitrypsin in the serum combines only with active trypsin. The units of trypsin activity inhibited are fairly comparable. However, the activity units depend on the particular assay conditions and the exact substrate concentration, which is further complicated by BAPNA's low solubility compared with its $K_m$.

Commercial trypsins are prepared from pancreatic extract and contain a mixture of (a) enzymatically inactive trypsinogen; (b) β-trypsin, a single-chain, enzymatically active protein derived from trypsinogen by removal of a six-amino-acid peptide from the amino terminus; (c) α-trypsin, a two-chain, enzymatically active protein derived from β-trypsin by peptide bond hydrolysis between lysine 131 and serine 132; and (d) various impurities and peptide fragments (20). Since Schroeder and Shaw (22) reported β-trypsin to be more active toward BAPNA than is α-trypsin, we were concerned that varying the α/β ratio in commercial trypsin preparations might affect the assay results. However, Table 2 indicates that α- and β-trypsin behave similarly in our assay when standardized on an active-site basis.

Fig. 4. Proposed automated method compared with (left) the manual trypsin inhibitory capacity method of Eriksson (11) and (right) the Beckman nephelometric α1-antitrypsin method.
The precision of the assay was evaluated over a period of about a year. Table 3 shows the within-rotor and day-to-day precision.

Comparison with a manual determination of trypsin inhibitory capacity and a nephelometric α₁-antitrypsin assay is shown in Figure 4.

The trypsin-inhibitory capacity of 250 blood donors was normally distributed, with a mean of 27 (SD 3.5) μmol of trypsin active sites inhibited per liter of serum. This mean compares well with the mean trypsin-inhibitory capacity we determined by Eriksson's (11) manual assay for our blood donors—about 1.0 g of trypsin inhibited per liter of serum (42 μmol of trypsin protein inhibited per liter of serum)—with use of Worthington trypsin and A₁₆₀₀ for trypsin quantitation (Worthington trypsin appears to be about 70% active). The mean automated trypsin-inhibitory capacity for blood donors also compares well with Beckman's reported mean normal value for α₁-antitrypsin, 1.6 g/L (29.6 μmol of α₁-antitrypsin per liter of serum).

In summary, we have described an automated method for functionally measuring serum antitryptic capacity with the Instrumentation Laboratory Multistat III. The assay is rapid, precise, and correlates well with an immunological assay for α₁-antitrypsin and with a manual trypsin-inhibitory capacity assay. The assay is calibrated in absolute terms by using a chromogenic trypsin active-site titrant and, as a result, is independent of trypsin supplier and relative purity. This standardized functional assay for serum antitryptic capacity should allow more meaningful interlaboratory comparisons and a better understanding of diseases involving protease inhibitor deficiency.

We thank Donald Koehler and Marcia Kershaw for technical assistance, Harold Markowitz and Marcy Schroeder for supplying sera phenotyped for α₁-antitrypsin, and Dana Evensen for manuscript preparation. This work was supported by Veterans Administration research funds.

References