A Fluorometric Determination of Trypsin-Like Amidase Activity and Activity of Trypsin Inhibitors in Serum

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The activity of crystalline trypsin and the trypsin-like amidase activity and the activity of trypsin inhibitors in serum were measured fluorometrically, with α-benzoyl-L-arginine-β-naphthylamide (BANA) as substrate. The method is simpler and more sensitive than the colorimetric method based on the Bratton-Marshall reaction. Serum of pancreatectomized dogs hydrolyzed BANA at a significant rate, which suggests that serum amidases other than trypsin are active in this determination.

PROTEOLYTIC AND RELATED ENZYMES, possibly of pancreatic origin, are reportedly present in human serum (1-4). Peptidase activities, including one that is trypsin-like, may be increased in blood during various pancreatic diseases (1-4). However, these proteolytic activities are still poorly characterized. Several methods for determining trypsin-like protease, amidase, and esterase activities in serum have been reported.

Proteins such as casein (5), hemoglobin (6), fibrin (7), and gelatin (8) are used as substrates for the determination of trypsin-like activity in serum. Synthetic substrates can also be used. The following compounds have been utilized as substrates for measuring amidase activity: α-benzoyl-L-arginine amide (9), α-benzoyl-L-arginine-β-naphthylamide (10, 11), α-benzoyl-L-arginine-p-nitroanilide (12-14), α-benzoyl-L-lysine hydrazide (15), α-carbobenzoxy-diglycyl-L-arginyl-2-naphthylamide (16, 17), β-carboxypropionyl-L-diarginy1-2-naphthylamide (16), and α-carbobenzoxy-L-triarginy1-2-naphthylamide (16).

For the measurement of esterase activity, α-benzoyl-L-arginine ethyl ester (3, 18, 19), p-toluene-sulfonyl-L-arginine methyl ester (20-24), and L-lysine ethyl ester (25) are used as substrates.

The presence of trypsin inhibitors in blood is also well recognized (26-29), and they can be determined by many methods. Casein (30-32), fibrin (7), hemoglobin (29), gelatin (33), α-benzoyl-L-arginine-p-nitroanilide (13, 34-36), and p-toluene-sulfonyl-L-arginine methyl ester (21), and α-benzoyl-L-lysine hydrazide (15) have been used as substrates.

In 1959, Riedel and Wuensch (10) determined the activity of crystalline trypsin, with α-benzoyl-L-arginine-β-naphthylamide (BANA) as the substrate. Blackwood and Mandel (11) used BANA for the determination of trypsin-like activity in various tissues; they colorimetrically measured the β-naphthylamine liberated from BANA by trypsin, by use of the Bratton-Marshall reaction.

In this study we report a fluorometric technique for the determination of β-naphthylamine. This method utilizes BANA as a substrate and can be used to determine either trypsin-like activity or the activity of trypsin inhibitors in blood and other biological systems. It is simpler and more specific than the colorimetric technique.

Materials and Methods

Reagents

α-Benzoyl-L-arginine-β-naphthylamide (BANA) [Japan Industrial Standard (JIS), special grade, Nakarai Chem. Co., Japan]. One mmol/liter of water. The solution is stable for at least one month if it is kept at 0°C.
Tris buffer [tris(hydroxymethyl)aminomethane, JIS special grade, Nakarai Chem. Co., Japan]. One mole per liter, pH 8.

Crystalline trypsin (Difco, specific activity 1:250). This is dissolved in 0.002M HCl containing 0.05 mole of CaCl₂ per liter.


Procedures

Determination of trypsin-like activity in serum.
In this method BANA is hydrolyzed by trypsin (or trypsin-like enzymes), liberating β-naphthylamine, which is measured fluorometrically.

A serum specimen, 0.3 ml, is mixed with 0.1 ml of Tris buffer, 0.5 ml of BANA solution, and 0.7 ml of water. The reaction mixture is incubated for 3 h at 37°C. It is then chilled to 0°C and 5 ml of cold 95% ethanol is added with thorough mixing. The protein precipitate is removed by centrifugation.

The fluorescence of the supernatant liquid is determined at an excitation wavelength of 295 nm and an emission wavelength of 400 nm. The fluorescence of β-naphthylamine standard is similarly measured in the presence of the same concentration of BANA as in the test samples, and the amount of β-naphthylamine liberated from BANA by the trypsin-like enzyme in the serum is calculated. (BANA significantly reduces the intensity of the fluorescence of β-naphthylamine; therefore, the standard calibration curve of β-naphthylamine must be prepared in the presence of BANA.) The activity of the enzyme is expressed as micrograms of β-naphthylamine liberated from BANA by 1 ml of serum during the 1-h incubation.

Determination of the activity of trypsin inhibitors in serum. For the determination of the activity of total trypsin inhibitors, 150 μg of crystalline trypsin is incubated with and without serum. Serum—5 and 7 μl, respectively—is added to mixtures of 0.5 ml BANA, 0.1 ml of Tris buffer, and 150 μg of crystalline trypsin in the solvent described above. The mixture is diluted to 1.6 ml by adding distilled water. The reaction mixture is incubated at 37°C for 3 h. After the incubation, 5 ml of cold 95% ethanol is added and mixed to stop the reaction. After the precipitated protein is removed by centrifugation, the fluorescence of the supernatant fluid is determined as before. The inhibition of trypsin by serum is calculated from the difference in the activity in tubes containing 5 and 7 μl of serum. The unit of activity is expressed as micrograms of β-naphthylamine (whose liberation from BANA is inhibited by the trypsin inhibitors) per milliliter of serum per hour of incubation.

For the determination of the heat-stable inhibi- tor, serum specimens are diluted 20-fold with water and the pH is adjusted to 4 with HCl. This diluted serum specimen is treated at 60°C for 20 min. Of this heat-treated diluted serum specimen, 0.2 and 0.4 ml—equivalent to 10 and 20 μl of original serum—are incubated with and without crystalline trypsin, as described above.

Two trypsin inhibitors have been demonstrated by electrophoresis of serum on paper or starch, the minor one being an α₂-globulin and the major one an α₁-globulin that is unstable to heat and low pH values. Mehl et al. (35) say that the heat-unstable trypsin inhibitors are completely inactivated at pH 4.0 and 60°C for 20 min, and that the heat-stable factor is stable under these conditions. We removed the heat-unstable inhibitor activity by heating the serum at 60°C for 20 min at a pH of 4.

Results

Optimum Fluorescence of β-Naphthylamine

β-Naphthylamine shows fluorescence at an emission wavelength of 400 nm with an excitation wavelength of 295 nm in solution at pH 5.0 to 9.0. The fluorescence was greatest in distilled water, ethanol, methanol, chloroform, and various buffers such as phosphate, borate, and Tris. It was stable at 37°C for 24 h, but was less at

![Fig. 1. Effect of pH on the action of crystalline trypsin or serum on the hydrolysis of BANA](image)

![Fig. 2. Effect of time on the action of crystalline trypsin and serum on the hydrolysis of BANA](image)
95°C. Treatment of β-naphthylamine with acetone, strong acid, or alkali abolished this fluorescence. Since the fluorescence of β-naphthylamine is influenced by strong acids, deproteinization of the reaction mixture with acids is inappropriate. This is why we used ethanol to deproteinize serum in the reaction mixture.

**Sensitivity and Specificity of Fluorescence Determination of β-Naphthylamine**

When the colorimetric method based on the Bratton-Marshall reaction was compared with the fluorometric method, the latter was 100 times as sensitive. BANA fluoresces maximally at an emission wavelength of 355 nm when excited at 295 nm; therefore, the fluorescence of BANA and β-naphthylamine can be well separated. The differences are detailed below.

**Recovery of β-Naphthylamine from Serum**

The recovery of β-naphthylamine from serum was measured at 37°C for as long as 6 h. It remained constant at about 80%.

**Action of Crystalline Trypsin and Serum on Hydrolysis of BANA**

*Effect of pH.* Crystalline trypsin and serum both hydrolyzed BANA maximally at pH 8.0 (Figure 1).

*Effect of time.* Under the conditions of the present investigation, the hydrolytic action of 10 μg of crystalline trypsin and 0.5 ml of serum on BANA increased with the duration of incubation. The units of activity of the enzyme and the time of incubation were linearly correlated over 24 h (Figure 2).

**Effect of crystalline trypsin and serum amounts.** Various amounts of crystalline trypsin were incubated with BANA under the described conditions. The amounts of trypsin and the amounts of β-naphthylamine liberated from BANA were linearly correlated (Figure 3). When increasing amounts of serum were incubated with BANA, the enzymatic action did not increase linearly (Figure 3). However, under the conditions of this method, the amounts of serum and the units of activity of enzyme showed a linear relationship when less than 0.3 ml of serum was used. With more than 0.5 ml, the relation was no longer linear, showing some inhibition of enzymatic action.

**Effect of BANA concentration.** When 30 μg of crystalline trypsin was incubated with increasing concentrations of BANA under the conditions of the present investigation, the amount of β-naphthylamine liberated from BANA also increased, but at concentrations of over 230 μmol/liter this increase was not observed. When 0.4-ml aliquots of serum were similarly incubated with increasing doses of BANA, enzymatic activity progressively increased up to a concentration of 90 μmol/liter; thereafter, no further increase was observed.

**Fluorescence Spectra of Reaction Mixtures of Crystalline Trypsin or Serum and BANA**

Typical fluorescence spectra of BANA and β-naphthylamine are presented in Figures 4 and 5.
5 Under the conditions described, BANA is maximally excited at 295 nm and fluoresces at 355 nm. On the other hand, \( \beta \)-naphthylamine is excited at 295 nm and fluoresces at 400 nm. As shown in Figure 6, the fluorescence spectrum of the reaction mixture of crystalline trypsin and BANA had two peaks of fluorescence, at 355 and 400 nm, after incubation. The fluorescence peak at 355 nm is produced by BANA and at 400 nm, by \( \beta \)-naphthylamine. The intensity of the fluorescence peak at 400 nm increased linearly with increasing amounts of crystalline trypsin. Similarly, when BANA was incubated with serum, two fluorescence peaks were observed at the emission wavelength of 355 nm and 400 nm. As the amounts of serum incubated were increased, the intensity of the fluorescence peak at the emission wavelength of 400 nm also increased (Figure 7). These observations strongly indicate that the present method determines...
Fig. 6. Fluorescence spectra of reaction mixtures of crystalline trypsin and BANA.

β-naphthylamine liberated from BANA by trypsin-like enzyme(s) in serum.

Effect of Serum on Crystalline Trypsin Action on Hydrolysis of BANA

When 30 μg of crystalline trypsin was incubated with increasing doses of BANA as in previous experiments, the amount of β-naphthylamine liberated was increased, but at concentrations over 230 μmol/liter, the liberation of β-naphthylamine was not increased. When 2 μl of serum was added to 30 μg of crystalline trypsin, the amount of β-naphthylamine liberated was markedly reduced. As the concentrations of BANA were increased, the liberation of β-naphthylamine also increased, but less β-naphthylamine was liberated than with trypsin alone (Figure 8).

When increasing amounts of serum were incubated with 50 μg of crystalline trypsin, maximum inhibition was observed with 4 μl of serum. Thereafter no inhibition occurred and the trypsin-like activity gradually appeared (Figure 9). However, when the serum was pretreated at pH 8.0, 55°C, for 30 min, less inhibition was observed than with untreated serum. In this case, maximum inhibition was obtained with 6 μl of serum; with more serum, no trypsin-like activity appeared, in contrast to the effect of untreated serum. If serum at pH 4.0 was heated to 55°C for 30 min, there was a further decrease in the inhibition of the activity of trypsin (Figure 9).

The inhibitory effect of serum on the trypsin action was measured with different amounts of trypsin. As seen in Figure 10, when 3 μg of crystalline trypsin was used, the inhibitory effect of serum was linear from 0.2 to 1.0 μl. If 30 μg of crystalline trypsin was used instead, the inhibitory effect of serum increased linearly from 1
to 2 µl, and when 300 µg of crystalline trypsin was used, the inhibitory effect of serum was linear between 2 and 20 µl. Accordingly, 100 to 150 µg of crystalline trypsin was used for the routine determination of trypsin inhibitors in serum.

Effect of Large Quantities of Serum on Crystalline Trypsin Action in the Hydrolysis of BANA

When 10 µg of crystalline trypsin was incubated with 0.5 ml of serum from healthy subjects, the hydrolysis of BANA by trypsin was reduced about 60%. However, the 10 µg of crystalline trypsin was added to the same serum that had been pretreated at either pH 2 or 11 for 1 h at room temperature, the hydrolytic action of

10 µg of crystalline trypsin was completely inhibited (Table 1).

The same treatment of serum resulted in marked inactivation of both trypsin-like activity and the activity of trypsin inhibitors in serum (Table 2). Despite the inactivation of most trypsin inhibitors, 10 µg of crystalline trypsin was also completely inactivated. However, the untreated serum, which had about four times as much trypsin inhibitory activity as the treated serum, did not inactivate 10 µg of crystalline trypsin completely.
Table 1. Effect of Serum on Hydrolysis of BANA by Crystalline Trypsin

<table>
<thead>
<tr>
<th>Addition</th>
<th>( \beta )-naphthylamine liberated, ( \mu g/h )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crystalline trypsin, 10 ( \mu g )</td>
<td>1.50</td>
</tr>
<tr>
<td>Human serum, 0.5 ml</td>
<td>0.25</td>
</tr>
<tr>
<td>Untreated</td>
<td>0.25</td>
</tr>
<tr>
<td>Treated at pH 2</td>
<td>0</td>
</tr>
<tr>
<td>Treated at pH 11</td>
<td>0</td>
</tr>
<tr>
<td>Crystalline trypsin, 10 ( \mu g ), untreated human serum, 0.5 ml</td>
<td>0.52</td>
</tr>
<tr>
<td>Crystalline trypsin, 10 ( \mu g ), human serum treated at pH 2, 0.5 ml</td>
<td>0</td>
</tr>
<tr>
<td>Crystalline trypsin, 10 ( \mu g ), human serum treated at pH 11, 0.5 ml</td>
<td>0</td>
</tr>
</tbody>
</table>

Effect of Time on Inhibition of Trypsin Activity by Serum

Hydrolysis of BANA by trypsin was inhibited by serum. This inhibition by serum increased linearly with time during incubation for 4 h under the conditions of this experiment.

Quantitative Study of Inhibition of Trypsin Activity by Varying Amounts of Serum

When 150 \( \mu g \) of crystalline trypsin was incubated with varying amounts of serum, a linear relationship between the amount of serum and the degree of inhibition of trypsin action was observed (Figure 11). For this reason, 5 and 7 \( \mu l \) of serum was used for the determination of total trypsin inhibitors. In the case of heat-stable trypsin inhibitors, 10 and 20 \( \mu l \) were selected.

Levels of Trypsin-Like Activity and Activity of Trypsin Inhibitors in Man and Dogs

The trypsin-like activity was greater in human serum than in dog serum, but the reverse was true for trypsin inhibitors (Table 3). Even the serum of totally pancreatectomized dogs showed a significant trypsin-like activity; this suggests that other proteolytic activities are also involved in this determination. (These sera were obtained one week to a month after the operation.)

Discussion

Trypsin is known to hydrolyze the amide and ester of arginine or lysine. According to Gullick (37), benzoyl-arginine amide is more sensitive to trypsin than to plasmin or thrombin. In this study, therefore, BANA was used as the substrate for determining trypsin-like activity in serum. However, the amidase activity in serum as determined by this method involves not only the activity of trypsin but also of other proteolytic enzymes, since the blood of the totally pancreatectomized dogs hydrolyzed BANA at a significant rate.

Table 2. Effect of Serum Pretreatment at pH 2 and 11 on Trypsin-Like Activity and Activity of Trypsin Inhibitors in Serum

<table>
<thead>
<tr>
<th>Addition</th>
<th>Trypsin-like activity; ( \beta )-naphthylamine liberated, ( \mu g/h )</th>
<th>Activity of trypsin inhibitors; inhibition of ( \beta )-naphthylamine liberation, ( \mu g/h )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum, untreated, 0.5 ml</td>
<td>0.25</td>
<td>671.66</td>
</tr>
<tr>
<td>Serum, pretreated at pH 2, 0.5 ml</td>
<td>0</td>
<td>95.85</td>
</tr>
<tr>
<td>Serum, pretreated at pH 11, 0.5 ml</td>
<td>0</td>
<td>95.85</td>
</tr>
</tbody>
</table>

Table 3. Trypsin-Like Activity and Activity of Trypsin Inhibitors in Serum of Man and Dogs

<table>
<thead>
<tr>
<th>Age, yr.</th>
<th>Trypsin-like activity; ( \beta )-naphthylamine liberated, ( \mu g/ml ) of serum/h</th>
<th>Total Inhibitor</th>
<th>Heat-stable Inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal subjects</td>
<td>Trypsin-like activity; ( \beta )-naphthylamine liberated, ( \mu g/ml ) of serum/h</td>
<td>Activity of trypsin inhibitors; inhibition of ( \beta )-naphthylamine liberation, ( \mu g/ml ) of serum/h</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>20-61</td>
<td>(0.63 ± 0.15)*</td>
<td>206±1,000</td>
</tr>
<tr>
<td>Female</td>
<td>20-26</td>
<td>(0.51±1.11)</td>
<td>243±800</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.85±0.20)</td>
<td>(471±147)</td>
</tr>
<tr>
<td>Normal dogs</td>
<td>0.29±0.74</td>
<td>880±1,600</td>
<td>267±732</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.49±0.19)</td>
<td>(1,183±238)</td>
</tr>
<tr>
<td>Totally pancreatectomized dogs</td>
<td>0.32</td>
<td>1,160</td>
<td>292</td>
</tr>
<tr>
<td></td>
<td>0.30</td>
<td>1,400</td>
<td>510</td>
</tr>
<tr>
<td></td>
<td>0.19</td>
<td>(1,280)</td>
<td>(401)</td>
</tr>
</tbody>
</table>

* Figures in parentheses indicate the mean ±SD.
The present technique is more sensitive and simple than the colorimetric procedure based on the Bratton-Marshall reaction. Furthermore, it is more specific, since fluorescent substances such as β-naphthylamine are absent from blood. Simultaneous determination of the trypsin-like activity and the activity of trypsin inhibitors with the same substrate, BANA, would be convenient for the study of the physiological significance of the proteolytic activity in blood. Other amide substrates (e.g., α-carbobenzoxy-diglycyl-L-arginyl-2-naphthylamide, β-carboxypropionyl-L-diarginy1-2-naphthylamide, and α-carbobenzoxy-L-triarginy1-2-naphthylamide) can also be used in this method. These substrates have been reported to be split more readily than BANA by trypsin (16).

It is of particular interest that, as the amount of serum incubated with crystalline trypsin was progressively increased, the inhibition of the action of crystalline trypsin was proportionally increased. However, further increases in the amount of serum resulted in the disappearance of the inhibiting effect of serum on the action of trypsin, and trypsin-like activity reappeared. This is probably caused by the formation of an exogenous crystalline trypsin-globulin complex which protects trypsin from the action of trypsin inhibitors, as the amounts of serum protein increase. Furthermore, when 10 μg of crystalline trypsin was added to 0.5 ml of serum, complete inactivation did not occur despite the fact that the serum contained a sufficient amount of trypsin inhibitor to inactivate 10 μg of crystalline trypsin. However, when 10 μg of crystalline trypsin was added, after the treatment of the serum at pH 2 and 20°C for 1 h, complete inactivation of crystalline trypsin by 0.5 ml of serum did occur. This strongly suggests that the treatment at pH 2.0 modified serum protein so that the formation of trypsin-globulin complex was impaired. The trypsin-like activity measured by the present method is, in part, a measure of proteolytic activity due to the trypsin-globulin complex, which is known to be uninfluenced by serum inhibitors (13, 14, 35).

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References