An Evaluation of Serum "Trypsin" Tests

Arthur L. Babson, Prunella A. Read Williams, and George E. Phillips

When crystalline trypsin was dissolved at various concentrations in pooled serum, the rates of hydrolysis of \(\alpha\)-benzoyl-l-arginineamide (BAA) and N-\(\alpha\)-benzoyl-dl-arginine-\(\beta\)-naphthylamide (BANA) were not linear functions of the trypsin concentration. Also, the plot of trypsin activity in relation to trypsin concentration differed with different sera. The apparent tryptic activity toward these substrates is felt to be a function of the concentration of serum trypsin inhibitor and, with BANA only, some unidentified proenzyme which is activated by trypsin. Therefore, it is concluded that it is not feasible to measure serum trypsin concentration with these substrates.

Synthetic substrates have been used for many years to elucidate the specific linkages hydrolyzed by various proteolytic enzymes and to study the kinetics of these reactions. In 1939 Bergman and his associates (1) found that \(\alpha\)-benzoyl-l-arginineamide (BAA) was readily hydrolyzed by crystalline trypsin to yield benzoyl-l-arginine and ammonia. More recently, the synthesis of N-\(\alpha\)-benzoyl-dl-arginine-\(\beta\)-naphthylamide (BANA) and its use to measure the activity of crystalline trypsin has been described (9). Nardi (5-7) has reported on the use of BAA to measure "trypsin" in serum as a diagnostic test for pancreatic disease, and both BAA and BANA have been proposed for this use by some chemical and reagent manufacturers. A consideration of the well-known presence of a powerful trypsin inhibitor in serum (2, 4, 8, 11) and the reported instability of trypsin at the pH of serum (2, 10) led us to investigate the validity of these tests.

Materials and Methods

The assays were carried out on trypsin (2X recrystallized, Nutritional Biochemicals Corp.) which was dissolved directly in serum. The serum was obtained from unselected hospital patients, and was either...
freshly pooled or had been pooled and frozen for periods up to several months.

**Procedure**

**Using BAA**

The method of Nardi (5) was followed with some modifications. He used the microtechnic of Schwert *et al.* (10) which was originally developed for studying the kinetics of trypsin activity. The extent of trypsic hydrolysis in 2 ml. of reaction mixture containing 0.1 mM of substrate at various times was determined by transferring a 0.2-ml aliquot to a Conway microdiffusion unit and titrating the ammonia formed with 0.01 N HCl. Since Nardi used a constant incubation time of 1 hour, we modified his technic in that the reaction was run in the Conway unit, and the ammonia formed in the entire reaction mixture was determined. Thus our titrations were increased tenfold, and the need for special microburettes was eliminated.

**Using BANA**

In a test tube warmed to 37°, 0.2 ml. of serum was added to 0.2 ml. of 0.1 M glycine buffer, pH 10.0, and 5 ml. of substrate (20 mg. BANA dissolved in 100 ml. H2O). After 30 min. the tube was removed from the water bath, and 1 ml. of 0.075 M tartaric acid was added followed by 1 ml. of a 0.1% solution of diazotized 5-nitro-o-anisidine (Fast Red Salt B; Matheson, Coleman and Bell). This solution was freshly prepared just prior to use and kept in ice. After 10 min. the absorbance was determined at 530 mμ. The absorbance of a control (determined by incubating the buffered substrate for 30 min. and then adding the tartaric acid, serum, and diazonium salt in that order) was subtracted from the absorbance of the sample.

**Results and Discussion**

Nardi (6) states that “the serum trypsin present in the original sample is proportional to the amount of ammonia produced and is expressed as units of trypsic activity.” However, he gives no evidence for this and does not define his unit. When we assayed for trypsin in pooled serum, we found no significant activity by the BAA method with trypsin concentrations below 2 mg./ml. When the trypsin concentration was increased above 2 mg./ml., activity was indicated by an increase in titration which was proportional to the increase in trypsin concentration (Fig. 1). The concentration of trypsin required to pro-
duce this change in slope varied with different serum pools and appeared to be 2-3 mg./ml.

With the colorimetric assay using BANA, we obtained a different curve every time we used a different serum pool (Fig. 2). The curves, however, were quite reproducible from day to day when the same serum was used. The curves obtained with different serum pools had variable initial slopes which tapered off and then increased abruptly. The final slope was essentially identical in every case. It was noticed that the initial slope appeared to be inversely correlated with the age of the serum. Serum Pools 1, 2, and 3 were about 9, 6, and 2 months old, respectively, whereas the others were essentially fresh. This observation suggested that the trypsin was activating some labile proenzyme in the serum. It was apparently not plasminogen, since no activity towards BANA could be demonstrated with fresh serum preincubated with as much as 3 mg. of purified urokinase per milliliter.

The final slope in all probability represented the actual rate of hy-
drolysis of the two substrates by trypsin. Further evidence for this interpretation was provided by the slope of the curves found for trypsin dissolved in 0.85 % NaCl (Fig. 1 and 2). Although the slope with BANA fell off in the higher concentrations, the initial slopes in physiological saline were essentially identical to the final slopes in serum for both BAA and BANA. The nonlinearity of the curve with BANA might be a function of autolysis of the trypsin, since the low concentration of the substrate in this assay would afford little protection against this phenomenon (10). The concentration of trypsin at the break in the curve probably was a measure of the concentration of serum trypsin inhibitor. This concentration of trypsin varied from 1.25 to 2.67 mg./ml. depending upon the serum pool—values which are in agreement with reported levels of serum trypsin inhibitor (2, 4, 8, 11).

To determine if the slight initial slope observed with the BAA procedure was identical with the initial slope found by the BANA method, various concentrations of trypsin in serum Pool 7 were assayed by both methods. Since the calculated ratio of the initial slope to the final slope was 0.30 for the BANA method and only 0.038 for the BAA pro-
procedure, it was concluded that the phenomenon responsible for the initial slope using BANA was only slightly operative, if at all, with BAA. It would appear that the activity Nardi found in serum from patients with pancreatitis and carcinoma of the pancreas was not due to trypsin. It is noteworthy that patients with these diagnoses were found by Homer et al. (4) to have abnormally high serum trypsin inhibitor capacities. These authors were also unable to demonstrate any activity by Nardi’s method in these sera. Similarly, Floch and Groisser (3) were unable to demonstrate activity toward benzoyl-1-arginine ethyl-ester in the sera of such patients.

Surprisingly, trypsin was found to be quite stable in serum (Table 1). The fact that the trypsin molecules were surrounded by other protein molecules and therefore had little opportunity to undergo autolysis probably accounts for the observed stability. That the trypsin was actually digesting protein was evident from the increase in TCA-soluble tyrosine, phenylalanine, and tryptophan, as evidenced by the increase in absorbance at 280 m\(\mu\).

### Table 1. Stability of Trypsin in Serum*

<table>
<thead>
<tr>
<th>Time at 27° (hr.)</th>
<th>0.01N HCl (mL)</th>
<th>O.D., 280m(\mu)</th>
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<tr>
<td>0</td>
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<tr>
<td>24</td>
<td>1.63</td>
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*A 3 mg./ml. solution of trypsin in Serum Pool 2 was allowed to stand at 27°. At intervals aliquots were assayed for activity by the BAA method and for hydrolysis of serum proteins by measuring the increase in absorbance at 280m\(\mu\) of the supernatant solution from a mixture of 0.5 ml. serum, 2.5 ml. water, and 2.0 ml. of 20% trichloroacetic acid.

References