A BIOLUMINESCENCE METHOD OF DETERMINING ANTIPROTEASE ACTIVITY

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UDC 616.153.1:577.152.344.042.2/-074

KEY WORDS: antiproteases; luciferase; luminescent bacteria.

Protease inhibitors are currently being intensively studied in biochemistry. In clinical practice determination of the antiprotease activity (APA) of the blood in a variety of pathological states can be used as an additional sign of diseases accompanied by tissue destruction (acute pancreatitis, peritonitis, myocardial infarction, hepatitis, cirrhosis of the liver, emphysema of the lungs, etc.), as many workers have noted [3, 6, 13].

The APA of specimens for study can be determined either by immunochemical methods [12] or by their ability to inhibit tryptic activity. For this purpose various protein substrates are used: hemoglobin [5], caseine [1, 10], fibrin [15], or synthetic low-molecular-weight substrates [7, 8]. The method of enzymic hydrolysis of benzolyl-D,L-arginine paranitroanilide (BAPNA), as the protease substrate, which is based on the formation of the colored product p-nitroaniline, is the most widespread. However, all known methods are distinguishable by the long time taken for analysis (from 60 min to 24 h), which discourages their widespread application to clinical practice.

The possibility of studying a proteolysis system by a bioluminescence method was demonstrated previously [14]. In this case bacterial luciferase is used as the protein substrate: its activity falls exponentially and the pseudofirst-order constant for inactivation of the enzyme is proportional to the quantity of active protease in the reaction mixture. The mechanism of interaction of luciferase with trypsin consists of splitting the α-subunit of luciferase into two fragments, with total loss of enzyme activity [9].

Bioluminescence analysis of protease activity does not involve any lengthy procedures and, for that reason, the writers have suggested its use for determination of antiprotease activity, with the aim of shortening the time of analysis.

EXPERIMENTAL METHOD

A preparation of luciferase from *Photobacterium leiognathi* (from the collection of the Institute of Biophysics, Siberian Branch, Academy of Sciences of the USSR, strain No. 54), purified by the method described previously [2], was used. Preparations of Gordox (Hungary), Contrykal (East Germany), and plasma from blood donors, diluted with 0.1 M Na-phosphate buffer in the ratio of 1:10, were used as antiproteases. Trypsin was obtained from Serva (West Germany), with specific activity of 40 units/mg.

The method involved preliminary incubation of antiprotease — 40 μl of a solution of Gordox (100 KIU/ml), 40 ml of a solution of Contrykal (100 AU/ml), or 40 μl of plasma — with 200 μl of a solution of trypsin (100 μg/ml) for 1-2 min at 30°C. An aliquot with a volume of 120 μl was then added to a reaction mixture containing 20 μl of a solution of luciferase and NADH:FMN oxidoreductase (FMN is the abbreviation for flavin mononucleotide; 0.3 mg protein to 1 ml of enzyme preparation), and also their substrates: 50 μl of 0.005% myristic aldehyde, 50 μl of $6.6 \cdot 10^{-5}$ M FMN, 360 μl of 0.1 M Na-phosphate buffer, pH 7.0, and 300 μl of $2.68 \cdot 10^{-4}$ M NADH. Bioluminescence was measured for 1 min in the constant-temperature cuvette of a luminometer of the authors' own design [4] (Fig. 1,1). In the control, 40 μl of 0.1 M Na-phosphate buffer was added to 200 μl trypsin (Fig. 1, 2).

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Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 100, No. 11, pp. 629-631, November, 1985. Original article submitted July 17, 1984.

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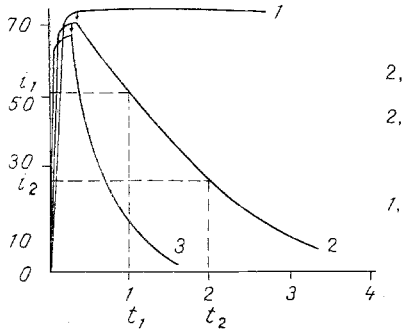

Fig. 1

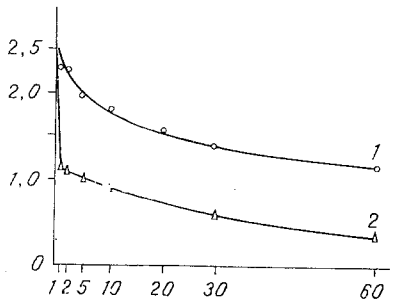

Fig. 2

Fig. 1. Time course of bioluminescence without additions (1), in presence (2) and in absence (3) of antiprotease. Abscissa, time (in min); ordinate, intensity of luminescence (in relative units). Arrow indicates time of addition of trypsin or of trypsin with antiprotease to reaction mixture.

Fig. 2. Effect of duration of incubation on constant of inactivation of luciferase by trypsin. Abscissa, time (in min); ordinate, inactivation constant (in min^{-1}). 1) control (trypsin + phosphate buffer), 2) experiment (trypsin + plasma). pH of solutions 7.0

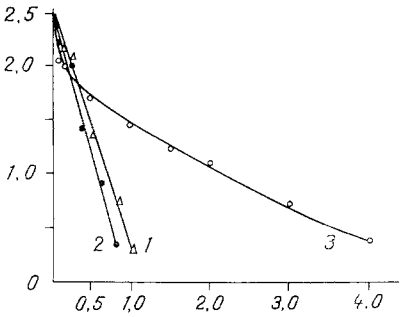

Fig. 3. Dependence of constants of inactivation of luciferase by trypsin on content of antiprotease. Abscissa, content of antiprotease (in µl per sample); ordinate, inactivation constant (in min^{-1}).

Thanks to an excess of protease in the incubation mixture, a reduction of fluorescence due to the action of the residual trypsin activity on luciferase is observed. The binding time of trypsin with its natural inhibitors does not exceed 1 min (Fig. 2). In known methods the incubation time is 10-30 min or more, which in our opinion, prolongs the investigation unjustifiably.

Residual protease activity is determined from the inactivation constant (K) of luciferase, which is calculated by the equation:

$$K = \frac{\ln i_1 - \ln i_2}{t_2 - t_1},$$

where i_1 and i_2 denote bioluminescence at times t_1 and t_2 respectively. APA is determined as the difference between the inactivation constants of luciferase in the presence and absence of the antiprotease.

EXPERIMENTAL RESULTS

The constant inactivation of luciferase by trypsin is directly proportional to the quantity of active trypsin [14], but the inhibitors bind trypsin irreversibly. Taking these facts into account it can be postulated that the inactivation constant is inversely proportional to the quantity of antiproteases, and this was confirmed by our results (Fig. 3: 1,2).

Hence APA can be measured as the quantity of bound trypsin. APA is expressed in milligrams of enzyme inactivated by 1 ml of the antiprotease preparation.

Blood plasma (serum) possesses APA, but the relationship between the constant of inactivation of luciferase by trypsin and the quantity of added plasma is different (Fig. 3:3). Nonlinearity is observed during determination of APA, both when protein substrates [1] and the method with BAPNA [11] are used.

Example of the Calculation. The sample contained 10 μg of trypsin, and 0.02 ml of a solution of Gordox (1:50) was taken for determination of APA. The difference between the inactivation constant in the control and experiment was 0.7 (2.5-1.8), which is equivalent to 2.8 μg of trypsin. Consequently, 1 ml of the whole solution of Gordox binds:

$$\frac{2.8 \cdot 50}{0.02} = 7000 \ \mu g \ .$$

In the case of Contrykal 1 ml of the whole solution binds about 8 mg of crystalline trypsin, but 1 ml of plasma binds 2.3-2.6 mg of trypsin.

The suggested method was used to determine APA in blood plasma from patients with various surgical diseases and justified itself as a sensitive, simple, and rapid method. It is possible to determine as little as 100 ng of antiproteases, and the analysis consists of only two stages, with a total duration of 3 min.

The bioluminescence method of determination of APA can be used both in accurate biochemical investigations and for rapid analysis in clinical practice.

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