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Short Communication

A sensitive high-performance liquid chromatographic assay for trypsin-like enzymes

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Introduction

There is an increasing interest in trypsin-like proteolytic enzymes of physiological significance. Indeed, although present in very small quantities, these enzymes play important roles in several biological reactions such as digestion, blood clotting, fibrinolysis, complement action, fertiiization and kinin formation [1]. Thus very sensitive microassays are necessary for monitoring and studying the processes in which these enzymes are involved.

Methods using proteins such as casein or haemoglobin as substrates are not recommended because of their lack of accuracy and reproducibility.

*N*α-benzoyl-L-arginine ethyl ester (BAEE) is one of the most widely used synthetic substrates. The rate of hydrolysis can be determined by different methods. The consumption of alkali required to neutralize the free carboxylic groups of *N*α-benzoyl-L-arginine (BA), the product of hydrolysis, can be measured [2]. This method is also recommended by the Commission on Pharmaceutical Enzymes of the FIP (Federation Internationale Pharmaceutique). The release of ethanol from BAEE can also be estimated by the reduction of NAD^+ with alcohol dehydrogenase and by monitoring the increase of absorbance at 366 nm [3] or 334 nm [4].

Kinetic measurements can also be performed at 253 nm [5] or the decrease in absorbance followed at 285 nm [6] by utilizing the difference in molar absorptivity between BA and BAEE.

Sensitive methods have been developed with fluorogenic substrates [7–8]. The present communication describes the measurement of the released BA at 235 nm by directly injecting the incubation medium on to a HPLC column. The separation achieved on the reversed-phase system avoids interference by the unhydrolysed substrate without long extraction procedures or chromogenic reactions.

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The method has been confirmed on FIP standard enzyme preparations of papain and trypsin supplied by the Commission on Pharmaceutical Enzymes, Centre for Standards.

Experimental

Apparatus

A Knauer high-performance liquid chromatograph was equipped with an injector Rheodyne model 7125 with a 20-μl loop and connected to a variable wavelength UV detector (Kontron Uvikon LCD 725) and a Perkin–Elmer strip chart recorder.

Materials

Hippuric acid, BA and camphor-10-sulphonic acid were purchased from Fluka A.R. chemische Fabrik (Switzerland); BAEE (as hydrochloride) and enterokinase from Sigma Chemical Co. (St. Louis, MO, USA); papain (1 mg = 0.349 FIP unit) and trypsin (1 mg = 42.2 FIP unit) were supplied by the Commission on Pharmaceutical Enzymes, Centre for Standards, State Univ., Gent (Woterslaan 12, B 9000 Gent, Belgium). Methanol HPLC grade was supplied by Hoechst (Riedel de Haen); sodium chloride, monohydrogen sodium phosphate, dihydrogen potassium phosphate, calcium chloride, hydrochloric acid, acetic acid, ethylenediaminetetra-acetic acid disodium salt (EDTA), cysteine hydrochloride, all analytical grade, by Carlo Erba (Milano, Italy).

All reagents were used without further purification. The methanol and water were filtered with suction through 0.45 μm filters before use.

Chromatographic conditions

The mobile phase was prepared by first dissolving 2.32 g of camphor-10-sulphonic acid in 650 ml of water; 10 ml of glacial acetic acid was then added and the solution was diluted to 1000 ml with methanol.

The operating conditions were: ambient temperature; flow rate 2 ml min^{-1}; detector wavelength 235 nm; chart speed 0.5 cm min^{-1}; sensitivity 0.05–0.2 a.u.f.s.

The 250 × 4.6 mm i.d. column was packed with 10-μm Lichrosorb RP 18 (Perkin–Elmer).

Internal standard stock solution. A solution of 2 mg ml^{-1} hippuric acid in water was used.

Reference standard solution. Thirty milligrams of BA and 20 mg of hippuric acid (internal standard) were dissolved in 16 ml of 0.5 M HCl and 33 ml of 0.08 M phosphate buffer (pH 7) and the solution diluted to 100 ml with water.

The solution was prepared daily or stored at −20°C.

Enzymatic assays

(a) *Papain.* Two hundred microlitres of a solution of about 0.2 FIP units ml^{-1} in 0.08 M phosphate buffer (pH 7) was activated by incubation at 25°C with an equal volume of a solution containing 20 mM cysteine HCl, 8 mM EDTA and 600 mM NaCl.

After 5 min the enzymatic reaction was started with 400 μl of substrate solution (0.06 M BAEE in water at pH 7). The reaction was stopped after 15 min (unless otherwise specified) by adding 200 μl of 0.5 M HCl and then 200 μl of internal standard solution (stock solution diluted 1 : 1, v/v).

(b) *Trypsin*. Two hundred microlitres of a solution of about 1.7 FIP units ml^{-1} in 0.08 M phosphate buffer (pH 7) was activated by incubation for 60 min at 25°C with an equal volume of a solution containing 0.24 FIP unit ml^{-1} of enterokinase in 20 mM $CaCl_2$ (pH 6).

After activation the reaction was started with 400 μl of BAEE substrate solution and stopped after 15 min with 0.5 M HCl as described for papain. Internal standard stock solution was also added. In both cases the zero time result was obtained by incubating a sample in the absence of the enzyme.

After centrifugation the samples were filtered through 0.45 μm Millex-HV filters (Millipore). Aliquots of the incubation medium (5–20 μl) were directly injected into a liquid chromatograph and the liberated BA was determined.

One unit of activity represents the amount of enzyme preparation that, under the specified conditions, hydrolyses 1 μmol of BAEE per min. The specific activity is the number of units per mg of enzyme.

Smaller samples can be used provided that the total volume is adjusted to 200 μl with phosphate buffer.

Calculations

The amounts of BA were calculated from the chromatograms from the following formula:

$$\text{BA} = \frac{h_1}{h_2} \times \frac{\mu g_1}{m}$$

where:

h_1 = peak height of BA in the sample
h_2 = peak height of internal standard in the sample
μg_1 = internal standard in the sample in micrograms

$$m = \frac{h_1'}{h_2'} \times \frac{\mu g_2'}{\mu g_1'}$$

where:

h_1' = peak height of BA in the standard solution
h_2' = peak height of internal standard in the standard solution
$\mu g_1'$ = BA in the standard solution in micrograms
$\mu g_2'$ = internal standard in the standard solution in micrograms

$$\text{Specific activity} = \frac{(\mu g\ S - \mu g\ To) \times 10^3}{278.3 \times 15 \times \mu g\ E}$$

where:

μg S = μg of BA in the sample
μg To = μg of BA at zero time
278.3 = molecular weight of BA
15 = incubation time, min
μg E = quantity of enzyme in the sample in micrograms.

Results and Discussion

The use of HPLC for the assay of proteolytic activity of trypsin-like enzymes has not been reported previously. The present procedure does not require pretreatment of samples.

In Fig. 1A a chromatogram of standard solution is shown. In Fig. 1B a chromatogram corresponding to "zero time" is reported. The small quantities of BA present in the substrate as an impurity or possibly formed during incubation, was calculated and subtracted from the values found in the sample incubation mixtures. In Fig. 1C a chromatogram of a sample is shown. No endogenous substances interfering with the determination of BA were detected.

The enzyme solution was freshly prepared daily; solutions of BAEE substrate and enterokinase activator were stored at −20°C and solutions of cysteine activator were prepared freshly because the compound is easily precipitated.

The recovery of BA added to the incubation medium was 99.5% ± 1.94% for papain and 99.3% ± 1.51% for trypsin, respectively, over the range 8–1.700 μg ml^{-1}. The relative standard deviation of three determinations for each sample analysed ranged between 0.9% and 3.8% for trypsin and 0.8% and 3.5% for papain (Table 1). BA is stable during the incubation period of 15 min at 25 or 37°C.

Figure 2A and B show the amount of hydrolysed product plotted against the quantity of enzyme added. For trypsin it can be seen that the relationship is linear over the range 0–350 mU of enzyme.

For papain, which is much less active than trypsin, linearity was studied in a narrower range of concentrations (0–200 mU). The papain preparation with lower activity was

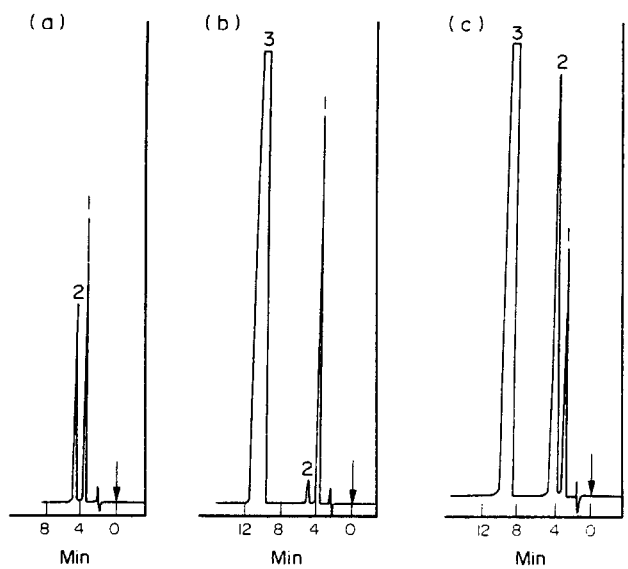

Figure 1
(A) Chromatogram of the reference standard solution containing hippuric acid 0.215 mg ml^{-1} (peak 1) as internal standard and BA 0.29 mg ml^{-1} (peak 2). a.u.f.s. = 0.1. (B) Chromatogram of the zero time solution. Key: (1) hippuric acid; (2) BA as impurity (53 μg); (3) BAEE as substrate. (C) Chromatogram of a sample (8 μg of trypsin) incubated as described in Experimental. Key: (1) hippuric acid; (2) BA liberated (1265 μg); (3) BAEE as substrate.

Table I
Percentage recovery of Nα-benzoyl-L-arginine

	Amount added (μg)	Amount found for papain (μg)	Amount* found for μg papain minus μg To	Recovery† %	Amount found for trypsin (μg)	Amount* found for μg trypsin minus μ To	Recovery† %
To	0	50.0	—	—	51.0	—	—
1	10	60.2	10.2 (3.5)	102.0	60.9	9.9 (3.8)	99.0
2	20	70.3	20.3 (2.8)	102.5	71.1	20.1 (3.2)	101.0
3	50	100.6	50.6 (1.2)	101.2	100.7	49.7 (1.4)	99.4
4	100	151.0	101.0 (1.3)	101.0	151.0	100.0 (1.3)	100.0
5	200	245.5	195.5 (1.1)	97.75	249.1	198.1 (1.8)	99.1
6	300	342.2	292.7 (2.2)	97.5	341.2	290.2 (1.5)	96.7
7	400	454.0	404.0 (1.4)	101.0	456.0	405.0 (1.2)	101.2
8	500	534.7	484.7 (2.1)	97.0	553.3	502.3 (1.3)	100.5
9	1000	1030.0	980.0 (1.2)	98.0	1021.0	970.0 (0.9)	97.0
10	2000	2020.0	1970.0 (0.8)	98.5	2021.0	1980.0 (1.2)	99.0
	Grand mean recovery %			99.5			99.3
	Grand relative standard deviation %			1.94			1.51

*Values in parentheses are relative standard deviations (%); $n = 3$.

†AB was added to the incubation medium composed as described under "Enzymatic assay", except that the enzyme was omitted. After 15 min incubation, 200 μl 0.5 M HCl and 200 μl of internal standard solution were added.

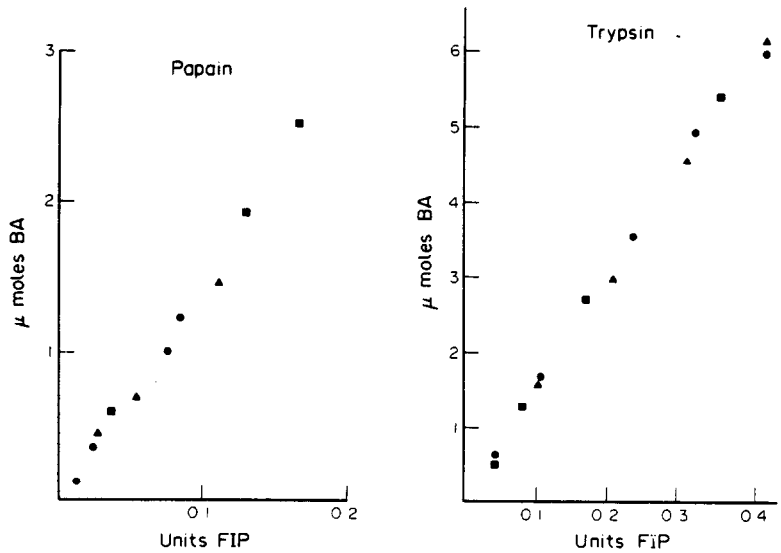

Figure 2
Correlation of BA concentration with the quantity of enzyme added. Various amounts of FIP papain (A) and trypsin (B) were incubated as described in the methods section and plotted against μ moles of BA produced during incubation for 15 min. Different symbols indicated that experiments were carried out on different days. The correlation coefficients (r) were 0.9994 for trypsin and 0.9947 for papain, respectively.

tested using a lower concentration of internal standard solution and increasing the sensitivity of the detector.

In order to confirm the validity of the method, the values of activity obtained were compared with those expressed in FIP units. For this purpose ten samples of papain with concentrations ranging between 12.5 and 250 μg ml^{-1} were analysed. The mean activity found was 0.357 units per mg with a relative standard deviation of 5%; the 95% confidence limits were 0.331–0.309.

For the ten trypsin samples analysed with concentrations ranging between 2.5 and 10 μg ml^{-1} the mean activity found was 41 units mg^{-1} with a relative standard deviation of 6%; the 95% confidence limits were 43.68–38.32.

As expected, the units of activity found with the present method are in good accord with those declared for the standard enzyme by FIP; the same conditions and the same substrate were used in the present work.

For low values of activity, the concentration of substrate can be reduced up to 10 times without affecting the linearity of the values obtained. As a consequence, the peak corresponding to zero time BA is reduced. In this way, it is therefore possible to obtain a sensitivity limit which is increased to 2 μg ml^{-1} by injecting 20 μl and operating the detector at an attenuation of 0.01 a.u.f.s. Such sensitivity values cannot be attained by using the concentration of substrate described under Experimental. In fact, if the sensitivity values were increased to 0.01 a.u.f.s., the peak corresponding to zero time BA would be too high to be subtracted significantly from the peaks corresponding to the BA of the analysed samples.

In conclusion, it is believed that this method is useful for both accurate bioassays and routine tests. The method may also have a technological interest and is presently used by

the authors to study the degradation with time of γ radiation-sterilized enzymes to be used in pharmaceutical formulations.

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