[Chem. Pharm. Bull. 23(5)1110—1113(1975)]

UDC 577.15.08 : 543.544

Assay of Arginine-esterase Activities by High Performance Liquid Chromatography[1)]

Kojiro Matsumoto, Toshio Imanari and Zenzo Tamura

Faculty of Pharmaceutical Sciences, University of Tokyo[2)]

(Received October 15, 1974)

Arginine-esterase activities of trypsin, plasmin and kallikreins were assayed by high performance liquid chromatography (HPLC) with BAEE (or TAME) and DAME.

1. In the case of conventional method with BAEE (or TAME), only the hydrolyzed product of BA (or TA) was eluted from the column of "Zipax" SCX (20 cm × 2.0 mm i.d.) with 0.1M phosphate buffer (pH 7.0), while BAEE (or TAME) was retained on the column. 10^{-3}—10^{-2} TAME units of these enzymes were easily measurable by determination of eluted product at several minutes intervals.

2. Using DAME as a fluorescent substrate, separation of DA from DAME was performed on the column of Hitachi gel #3010 (15 cm × 2.0 mm i.d.) by elution of 20% (v/v) Tris-HCl buffer (0.05M, pH 8.5) in methanol. In this condition, 4 pmole of DA was determined and 10^{-5}—10^{-4} TAME units of these enzymes were measurable.

Chemical assays of trypsin, plasmin, kallikrein *etc.* have been performed by use of synthetic substrates such as N-*p*-toluenesulfonyl-L-arginine methyl ester (TAME) and N-benzoyl-L-arginine ethyl ester (BAEE). Among of them, the simple and available assay at any laboratory is UV-method,[3)] based on measuring the increase of absorbancy due to the hydrolyzed product in the presence of the substrate. But as the product is not separated, this method is insufficient in sensitivity and reliability to assay minor and crude esterases presented in biological fluids such as in urine.

In this work, we applied high performance liquid chromatography (HPLC) to separative determination of hydrolyzed product of TAME or BAEE. Moreover, a sensitive assay was developed using 1-dimethylaminonaphthalene-5-sulfonyl-L-arginine methyl ester (DAME) as a fluorescent substrate.[4,5)]

Materials and Apparatus

Substrates——N-Benzoyl-L-arginine ethyl ester hydrochloride (BAEE) and N-*p*-toluenesulfonyl-L-arginine methyl ester hydrochloride (TAME) were purchased from Tokyo Chemical Industry Co., Ltd. 1-Dimethylaminonaphthalene-5-sulfonyl(dansyl)-L-arginine methyl ester (DAME) was synthesized from dansyl-L-arginine (DA, from Seikagaku-Kogyo Co., Ltd.) by methylation in absolute methanol with hydrogen chloride gas. TAME and BAEE were dissolved in 0.05M Tris-buffer (pH 8.0) containing 0.01M $CaCl_2$ and in the case of DAME, gelatin was added to the buffer at the concentration of 0.5%.

Enzymes——Trypsin, with 180 TAME units/mg and 90% protein, was purchased from Worthington Biochemical Co., Ltd. The enzyme was dissolved at the level of 1 mg/ml in 0.001M HCl. Lyophilized plasmin KABI (human) with 11 CU/mg was purchased from Seikagaku-Kogyo Co., Ltd. The enzyme was dissolved at the level of 0.9 mg/ml (*ca.* 1 TAME unit/ml) in 50% glycerol. Plasma kallikrein (human) with 50 TAME units/A_{280} and urinary kallikrein (human) with 3.5 TAME units/A_{280} were gifts from Dr. J.J. Pisano, National Institutes of Health (U.S.A.). Both enzymes were dissolved at the level of 10 TAME units/ml in the buffer containing 0.5% gelatin. These enzyme solutions were stored in a refrigerator. Before use, these solutions were diluted to the desirable concentration with the buffer containing 0.5% gelatin, if necessary.

1) Presented at the 94th Annual Meeting of Pharmaceutical Society of Japan, Sendai, April, 1974.
2) Location: *Hongo, Bunkyo-ku, Tokyo.*
3) B.C.W. Hummel, *Can. J. Biochem. Physiol.*, 37, 1393 (1959).
4) Z. Tamura, T. Nakajima, T. Nakayama, J.J. Pisano and S. Udenfriend, *Anal. Biochem.*, 52, 595 (1973).
5) Presented at the 90th Annual Meeting of Pharmaceutical Society of Japan, Sapporo, August, 1970.

Column——"Zipax" SCX (20 cm × 2.0 mm i.d.) from Dupont Co., Ltd. or Hitachi gel #3010 (15 cm × 2.0 mm i.d.) from Hitachi Seisakusho Co., Ltd. was packed in a glass tube (from Kyowa-Seimitsu Co., Ltd.).

Pump——The mini-micro pump model SU-1 from Kyowa-Seimitsu Co., Ltd. was used.

Detector——UV-detector LC-1 (Iatron, at 254 nm) or Aminco Fluorocolorimeter (No. 4-7439) was used as a detector. The latter was equipped with the ultraviolet lamp (No. 4-7125, maximum output at 360 nm), the primary filter (No. 4-7113, peak wavelength, 360 nm) and the secondary filter (No. 4-7116, sharp cut-off filter, 415 nm and above).

Recorder——Electronic Polyrecorder model EPR-2TC from Toa Electronics Ltd. was used.

Results

Assay of Trypsin Activity with BAEE and TAME

In this work, it is very important to examine the separation of the product from BAEE or TAME, since the incubation mixture contains an excess amount of substrate and a small amount of product.

N-Benzoyl-L-arginine (BA) or N-*p*-toluenesulfonyl-L-arginine (TA) was eluted from the column of "Zipax" SCX under the condition described in Fig. 1. In this condition, BAEE and TAME were retained on the column, and repeated injections of incubation mixture gradually shortened the retention times of BA and TA. However, as far as the peak area was concerned, the value was proportional to the quantity of BA or TA in a range of 0.5—10 nmoles or 2—30 nmoles, respectively, and more than 60 samples were assayed without washing away the substrates from the column.

When the column condition became worse, it was regenerated easily by washing with 10 ml of 0.1N NaOH and 20 ml of the buffer.

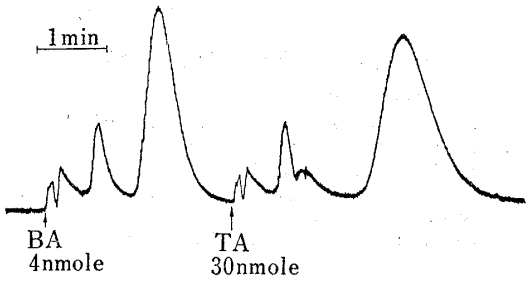

Fig. 1. Typical Elution Chromatogram of BA and TA

operating conditions
column: "Zipax" SCX (20 cm × 2.0 mm i.d.)
column temperature: room temp. (20°)
mobile phase: 0.1M phosphate buffer (pH 7.0)
flow rate: 1.5 ml/min
detector: UV-detector LC-1 (Iatron at 254 nm)

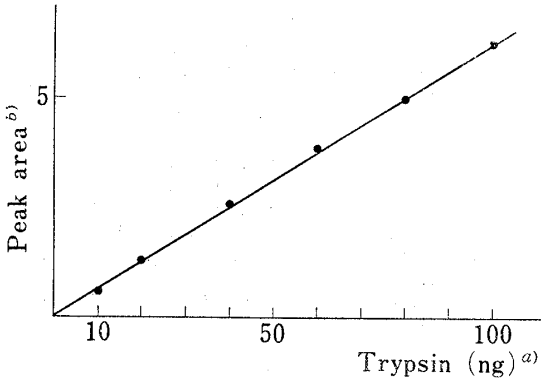

Fig. 2. Assay of Trypsin Activity with BAEE

a) 180 TAME units/mg, 90% protein from Worthington Biochemical Co. Ltd.
b) Blank value was substracted.

Activity of trypsin was measured by the following procedure. Ten μl of trypsin solution (1.0—10 μg/ml) was added to 90 μl of substrate solution (3.81 mg BAEE/ml, or 22 mg TAME/ml in Tris-HCl buffer) and incubated at 30° for 30 min. After addition of 30 μl of 0.75M $HClO_4$ to stop the enzyme reaction, a certain volume (3—10 μl) of the mixture was submitted to chromatography as described above. As shown in Fig. 2, a good correlation of peak area (half width × peak height) to trypsin activity in the range of 10—100 ng of trypsin was obtained using BAEE as a substrate.

Assay of Trypsin Activity with DAME

The relative fluorescent intensity of dansyl-amino acids was markedly reduced by water content in methanol,[6] therefore methanol containing 20% (v/v) water was used for a mobile phase.

6) Z. Tamura and T. Nakajima, *Protein, Nucleic acid and Enzyme*, 12, 729 (1967).

On the column of Hitachi gel #3010 (15 cm×2.0 mm i.d.), the retention time of DAME varied with pH of the mobile phase, while that of DA was almost unaffected as shown in Fig. 3. From the figure, 20% (v/v) Tris-HCl buffer (0.05M, pH 8.5) in methanol was selected as a mobile phase, considering sufficient separation and time saving. As little as 4 pmoles of DA was determined by measuring the peak height as shown in Fig. 4.

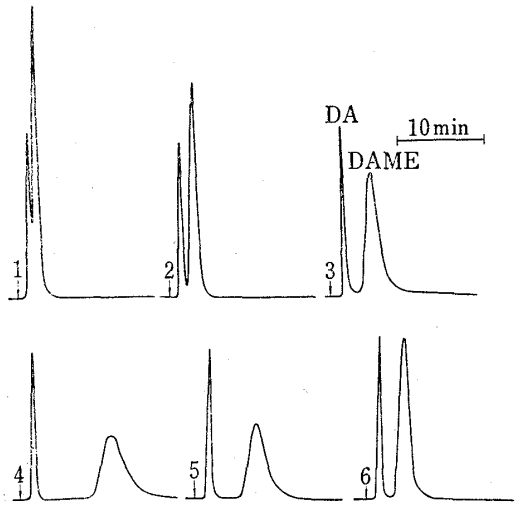

Fig. 3. Effect of pH on Separation of DA and DAME

operating conditions
column: Hitachi gel #3010 (15 cm×2.0 nm i.d.)
column temperature: room temp. (20°)
flow rate: 1.6 ml/min
detector: Aminco Fluorocolorimeter
mobile phase
1. 20% Tris-HCl buffer (0.05M, pH 7.0) in MeOH
2. 20% Tris-HCl buffer (0.05M, pH 8.0) in MeOH
3. 20% Tris-HCl buffer (0.05M, pH 8.5) in MeOH
4. 20% Tris-HCl buffer (0.05M, pH 9.0) in MeOH
5. 20% Glycine-NaOH buffer (0.05M, pH 10.0) in MeOH
6. 20% Glycine-NaOH buffer (0.05M, pH 11.0) in MeOH

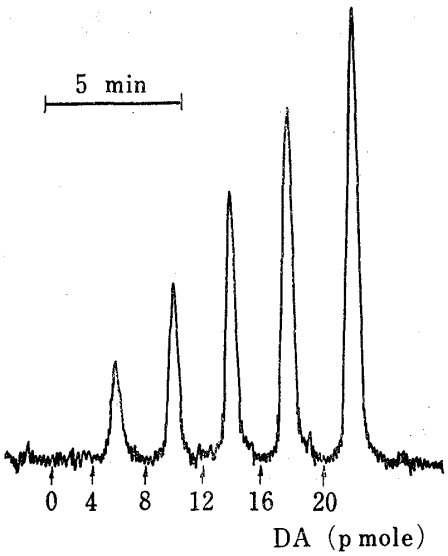

Fig. 4. Determination of DA

operating conditions
column: Hitachi gel #3010 (15 cm×2.0 mm i.d.)
column temperature: room temp. (20°)
mobile phase: 20% Tris-HCl buffer (0.05M, pH 8.5) in MeOH
flow rate: 1.6 ml/min
detector: Aminco Fluorocolorimeter

Trypsin activity was measured with the similar procedure to those with BAEE or TAME, but gelatin was added to the sample solutions so as to increase both solubility of DAME and stability of the diluted trypsin. Fifty μl of substrate solution in methanol (2.0 μmoles/ml) was evaporated to dryness under reduced pressure. Just before use, the residue was dissolved in 90 μl of the buffer containing gelatin and 10 μl of trypsin solution (10—100 ng/ml) was added. After incubation at 37° for 90 min, 30 μl of 0.75M $HClO_4$ was added to stop the enzyme reaction. A certain volume (3—10 μl) of the mixture was submitted to chromatography as described above. As shown in Fig. 5, 0.1—1.0 ng of trypsin was measurable.

Correlation of TAME Units to DAME Hydrolysis

The rate of hydrolysis of TAME was measured by our separative assay method with TAME. One unit was defined the amount of enzyme which hydrolyzed one μmole of TAME per minute at pH 8.0 and 37°.

Ten μl of about 10^{-3}—10^{-2} TAME units/ml of four enzyme solutions such as trypsin (bovine), plasmin (human), plasma kallikrein (human), urinary kallikrein (human) was assayed with DAME. As shown in Fig. 6, a good correlation of peak height to these enzyme activities in the range of 10^{-5}—10^{-4} TAME units was obtained, while the relative hydrolysis rates of DAME with these enzymes were different from each other.

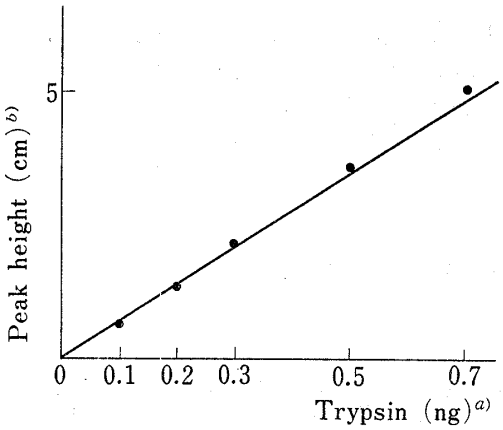

Fig. 5. Assay of Trypsin Activity with DAME

a) 180 TAME units/mg, 90% protein from Worthington Biochemical Co. Ltd.
b) Blank value was substracted.

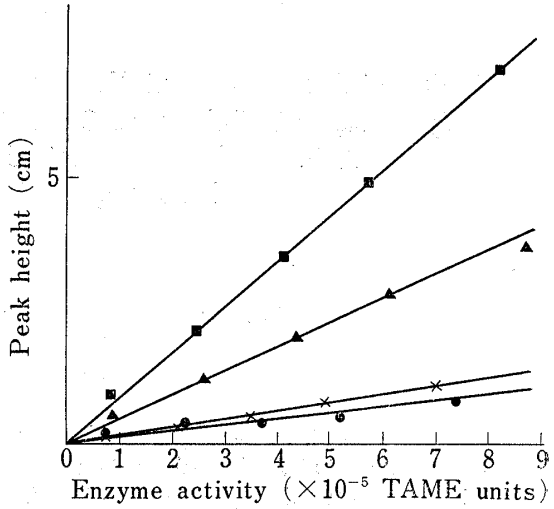

Fig. 6. Correlation of TAME Units to DAME Hydrolysis

—●—: trypsin
—×—: plasmin
—▲—: human plasma kallikrein
—■—: human urinary kallikrein

Discussion

Our separative assay method with TAME or BAEE is more selective than the conventional UV-method,[3] owing to the separative determination of the hydrolyzed product. The injection could be repeated to measure the products at several minute intervals, while accumulated substrates on the column were gradually eluted without significant shift of base line.

By this method, 10^{-3}—10^{-2} TAME units of trypsin can be assayed. In this experiment, we used UV-detector, at 254 nm but if the optimum wavelength be used for detection, the sensitivity for measuring TA and BA could be increased several times.

The method is very simple, since any color reaction is not required and would be generally applied to the other enzyme assays by the use of synthetic substrates with UV-absorption.

In the case of DAME as a fluorescent substrate, separative determination of the product required about ten minutes. DAME was stable for months in methanol in a freezer. But for the measurement of the product, blank value should be substracted because a trace of DA is usually concomitant with DAME. Similarly to trypsin, other arginine-esterases can be assayed as shown in Fig. 6. The sensitivity is much more increased to assay 10^{-5}—10^{-4} TAME units. So the method would be almost equal to the method with radioactive substrate[7] in its sensitivity and could assay human urinary kallikrein directly.

7) V.H. Beaven, J.V. Pierce and J.J. Pisano, *Clin. Chim. Acta*, 32, 67 (1971).