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Note

High-performance liquid chromatographic method for the determination of the esterase activity of subtilisin and kallikrein

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Subtilisins (E.C. 3.4.21.14) are alkaline serine proteinases originating from various strains of Bacillus[1], which have found industrial use as enzyme detergents. The enzymes of this class also show an hydrolytic activity towards esters of amino acids with aromatic residues or long aliphatic side-chains. Their catalytic activity can be measured spectrophotometrically with *N*-benzoyltyrosine ethyl ester as a substrate, or by titrating the hydrogen ions produced in the hydrolysis of *N*-4-toluenesulphonyl-L-arginine methyl ester[2] and of benzoyl-L-arginine ethyl ester (BAEE)[3].

Kallikrein (E.C. 3.4.21.35) is a serine proteinase occurring in various tissues and body fluids[4], which may be an important factor in the regulation of blood pressure, kidney function and glucose metabolism[5]. Among various methods reported for the determination of kallikrein activity, two are commonly applied: (a) a potentiometric method with BAEE as a substrate[6]; (b) a colorimetric method with D-Val-Leu-Arg-4-nitroanilide as a substrate[7].

As BAEE is a suitable substrate for both these enzymes, the high-performance liquid chromatographic (HPLC) procedure recently described for trypsin[8] has been extended to subtilisin and kallikrein, and the results were compared with those obtained by the titrimetic method.

EXPERIMENTAL

Materials

Subtilisin, soy bean trypsin inhibitor, benzoyl-L-arginine ethyl ester hydrochloride (BAEE · HCl) and benzoyl-L-arginine (BA) were all from Sigma (St. Louis, MO, U.S.A.); kallikrein was from Protogen (Laufelfingen Switzerland) and adenosine from Boehringer (Mannheim, F.R.G.). Acetonitrile was of HPLC grade (J. T. Baker, Deventer, The Netherlands). Water was deionized and distilled, and then filtered through a 0.45-μm membrane (Type HA; Millipore, Bedford, MA, U.S.A.).

Stock solutions

(1) Substrate: 0.2 *M* BAEE · HCl in 0.1 *M* Tris–HCl buffer pH 8. (2) Internal standard solution: 0.05 m*M* adenosine in 0.05 *M* hydrochloric acid. (3) Enzyme: 1

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TABLE I

ASSAY CONDITIONS

	Kallikrein	*Subtilisin*
Reaction		
Buffer	0.1 *M* Tris 0.25% (w/v) TI 0.2 m*M* EDTA	0.1 *M* Tris
pH	8.0	8.0
Substrate (BAEE) concn. (m*M*)	20	50
Temperature (°C)	25	37
Enzyme concn. (μg/ml)	1–7	5–60
Assay volume (ml)	2	2
Quencing of reaction		
Dilution	1/10	1/5
Final pH	3.5	2.6
Concn. of internal standard (m*M*)	0.024	0.024

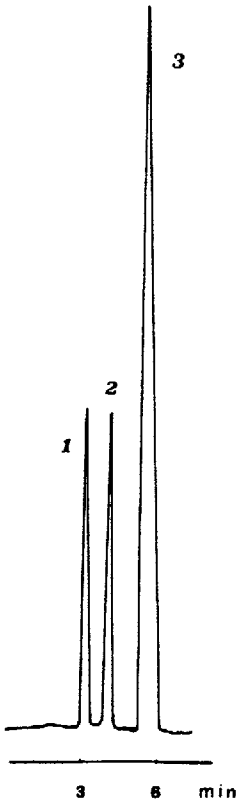

Fig. 1. Typical chromatographic pattern of adenosine (1), BA (2) and BAEE (3). Conditions: column, μBondapak C_{18} (30 cm × 4 mm I.D.); eluent, 10 m*M* sodium dihydrogenphosphate buffer pH 2.8–acetonitrile (73:27); flow-rate, 1 ml/min; detection, UV 254 nm.

mg/m subtilisin, 1.17 mg/ml kallikrein in 0.001 *M* hydrochloric acid. (4) Soy bean trypsin inhibitor (TI): 0.50% (w/v) in 0.1 *M* Tris–HCl pH 8.

Procedure

The reaction was started by adding known amounts of the enzymes to the substrate solution. At 3-min intervals, 20 μl of the mixture were diluted in the internal standard solution in order to stop the reaction and to obtain the appropriate con-

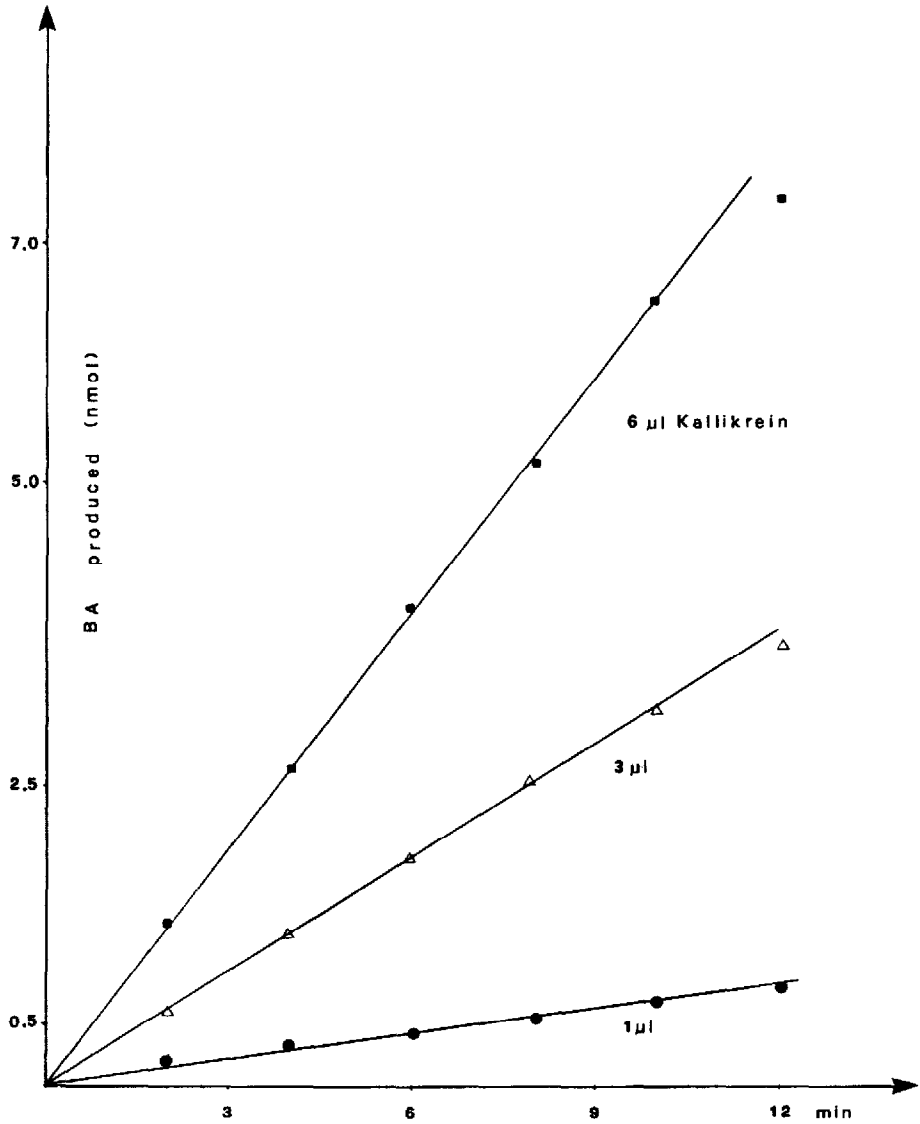

Fig. 2. Enzyme kinetics of kallikrein.

centration of the product (BA). Then replicate injections of 10 μl were made in the HPLC apparatus.

Table I gives a summary of the assay conditions.

Chromatographic conditions

The product, BA, was separated from the substrate, BAEE, and the internal standard by means of a Waters HPLC system, consisting of a Model 590 pump, a Model U6K universal injector, a Model 440 ultraviolet detector and a Model 730 Data Module. The column was a μBondapak C_{18} (300 mm $\times$ 3.9 mm, Waters Assoc.) with a pre-column of C_{18} Corasil (35 $\times$ 3.9 mm, Waters Assoc.). Elution was

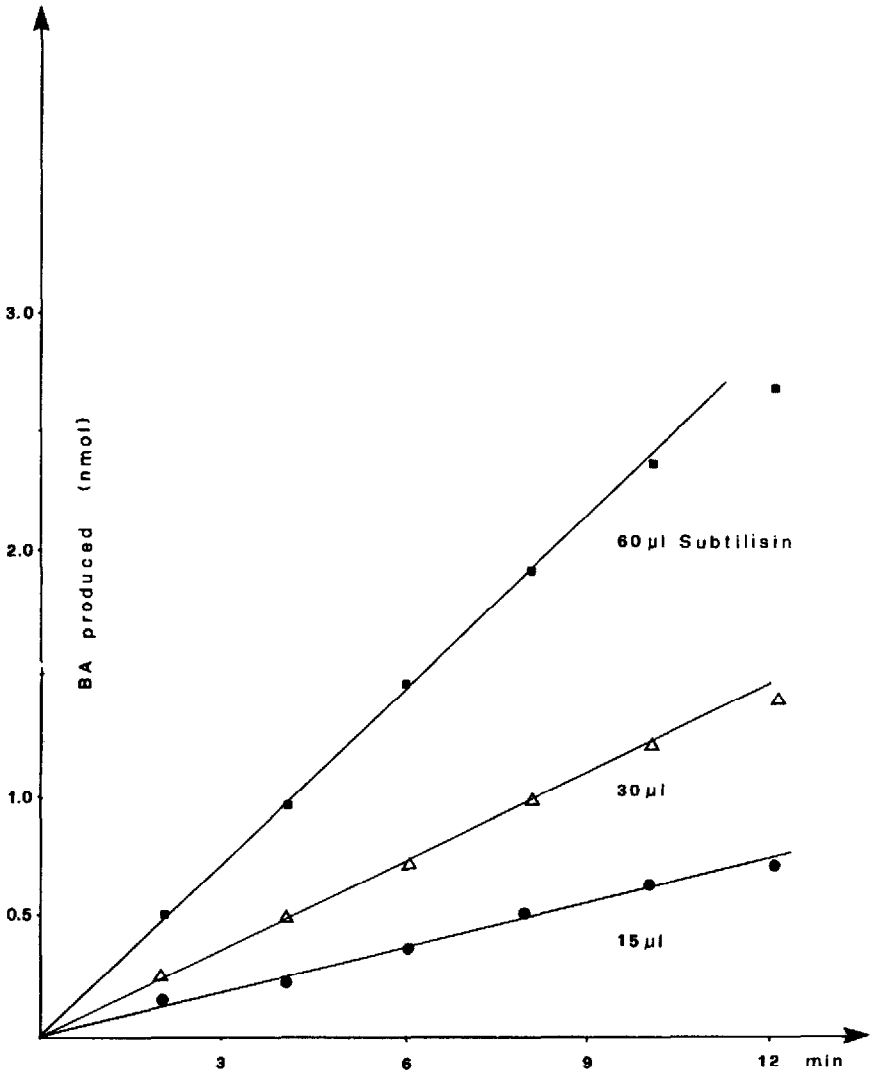

Fig. 3. Enzyme kinetics of subtilisin.

performed with 10 m*M* sodium dihydrogenphosphate buffer (pH 2.8)–acetonitrile (73:27) at a flow-rate of 1 ml/min and the effluent was monitored at 254 nm (0.05 a.u.f.s.).

RESULTS AND DISCUSSION

Fig. 1 shows a typical elution pattern where the product, BA, is clearly separated from the substrate, BAEE, and the internal standard.

Linear responses of the peak area ratios of BA and BAEE to the internal standard *versus* injected amounts were obtained with five replicate injections of standard solutions of BA and BAEE in the range of 0.1–6.0 nmol, as previously reported[8].

Time-course experiments showed that the rate of hydrolysis was linear within 10 min under the conditions described (Figs. 2 and 3).

From these control experiments an incubation time of 3 min was adopted for routine one-point enzyme assays. Therefore subtilisin and kallikrein at various concentrations were incubated with 0.05 *M* BAEE of 3 min, and the mixture was analyzed by HPLC. The initial rate of hydrolysis of BAEE catalyzed by the enzymes was proportional to their concentration in the range of 5–30 μg for subtilisin and 2–7 μg for kallikrein. Under these conditions the detection limits of subtilisin and kallikrein were as little as 7 and 1 μg/ml, respectively.

The specific activities of subtilisin or kallikrein were calculated as the rate of formation of BA using the equation

$$\text{Specific activity } (\mu\text{mol/min} \cdot \text{mg}) = \frac{a - 0.002}{b}$$

where a = μmol/min of BA produced overall, 0.002 = μmol/min of BA produced by spontaneous hydrolysis[8] and b = mg of enzyme.

According to the procedure described, commercial samples of subtilisin and kallikrein were assayed and the results were in good agreement with those obtained by the titrimetric method[9], the differences being of the order of 1.8%, likely due to the buffering action of the medium (standard deviation = 2.7%).

It can be concluded that the HPLC procedure described allows the determination of the esterase activity of subtilisin and kallikrein, and represents a valuable alternative to the titrimetric method.

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