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Assay for proteolytic activity using a new fluorogenic substrate (peptidyl-3-amino-9-ethyl-carbazole); quantitative determination of lipopolysaccharide at the level of one picogram

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A new sensitive fluorimetric assay has been developed using peptidyl-3-amino-9-ethyl-carbazole as substrate. The fluorescence intensity of free 3-amino-9-ethyl-carbazole (AEC) at 460 nm is between two and three orders of magnitude higher than the fluorescence intensity of acyl-AEC. The release of AEC from a peptidyl derivative by proteases may be monitored continuously during the hydrolysis step or may be quantified upon addition of a general inhibitor such as benzamidinium chloride. Using N-benzoyl-arginyl-AEC as substrate, as little as 1 ng trypsin may be detected. Using t-butyloxycarbonyl-Val-Leu-Gly-Arg-AEC and the amoebocyte lysate of *Limulus polyphemus*, as little as 1 pg lipopolysaccharide can be detected. This fluorimetric method allows detection of trace amounts of lipopolysaccharide (endotoxins) in various biological materials, including sera.

Key words: 3-amino-9-ethyl-carbazole/endotoxin/fluorimetry/lipopolysaccharide/proteases

Introduction

Bacterial lipopolysaccharide is easily detected using a lysate prepared from amoebocytes in the haemolymph of the horseshoe crab *(Limulus polyphemus* or *Tachypleus tridentatus)*. This lysate contains a proclotting enzyme and a clottable protein (coagulogen) (Solum, 1973; Murer *et al.*, 1975). Minute quantities of gram-negative bacterial lipopolysaccharide (Levin and Bang, 1964, 1968; Young *et al.*, 1972) activate the clotting enzyme to coagulate the protein. The so-called "Limulus test" exploits this gelation reaction for the detection of lipopolysaccharide (Yin *et al.*, 1972; Hochstein *et al.*, 1973). The sensitivity has been increased by using as alternatives to coagulogen (Nakamura *et al.*, 1976a, 1976b; Tai *et al.*, 1977), chromogenic (Nakamura *et al.*, 1977; Iwanaga *et al.*, 1978), and fluorogenic substrates. The latter includes various peptidyl derivatives of amino-methyl-coumarin (Zimmerman *et al.*, 1976) or methoxy-naphthylamine (Morita *et al.*, 1977). The basic requirements for the fluorogenic substrates are a relatively high quantum yield of the free amine, and the highest possible shift in the emission and/or excitation spectrum upon hydrolysis of the acyl derivative to the free amine; 3-amino-9-ethyl-carbazole (AEC) fulfills these requirements.

In this paper we describe the preparation of aminoacyl and peptidyl derivatives of AEC, their spectroscopic properties, and their use as fluorogenic substrates for proteases. In particular t-butyloxycarbonyl (tBOC)-Val-Leu-Gly-Arg-AEC as a fluorogenic substrate allows the detection of lipopolysaccharide at the level of 1 pg.

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Results

Spectral characteristics of AEC and of its acyl derivatives

The maximum absorbances of AEC and of its acyl derivatives are at 360 nm ($\epsilon_{M,1cm}$: 2500) and at 340 nm ($\epsilon_{M,1cm}$: 2200), respectively.

The structure of AEC and the hydrolytic reaction leading to its production are shown in Figure 1. N-benzoyl-arginyl-AEC was used as a substrate of trypsin and the spectra display an isosbestic point at 352 nm (Figure 2) and suggest the applicability of the compound as a chromogenic substrate to monitor hydrolytic activity of a related enzyme. At 370 nm, the ratio between the absorbances of the free and of the acylated AEC is maximal (12-fold). However, the fluorescence spectra of the free and acylated AEC shown in Figure 3 show even more dramatic differences. Indeed, when the free and acylated AEC are excited at the isosbestic point (λ : 352 nm), the maximum intensity of the acylated compound is located at 390 nm with a value of 0.12 while the maximum intensity of the free AEC is located at 460 nm with a value of 1.00. Furthermore, at 460 nm the relative fluorescence intensity of the acylated compound is only 0.006. Therefore, with excitation at 352 nm the fluorescence intensity of the acyl compound is ~ 0.6% that of the free AEC and <0.1% with excitation at 370 nm. The maximal fluorescence intensities of the acylated and free compound were obtained with excitation at their maximal absorbance wavelengths, 340 and 360 nm, respectively (Figure 3). However, more accurate results are obtained with excitation at the isosbestic point. The fluorescence intensity of AEC depends on the pH (Figure 4), the maximal intensity arising in the range pH 7–10. Therefore, the peptidyl-AEC may be used to monitor the hydrolytic activity of a very large number of proteases provided that the peptide moiety has the appropriate specificity. At lower pH, the fluorescence intensity of AEC is quenched due to ionisation of the amino group (pK = 5.95). Therefore, since enzyme activity cannot be stopped by lowering the pH, an inhibitor, benzamidine, was employed. However, as shown in Figure 5, benzamidine quenches the fluorescence of AEC; the quenching effect can be countered by the addition of N-dimethylformamide (DMF) up to 40% (vol/vol). If benzamidine is added with DMF to an AEC solution at final concentrations of 10 mM and 20% respectively, the resulting fluorescence intensity of AEC is slightly increased.

Hydrolytic enzyme assays

N-benzoyl-arginyl-AEC has been used to monitor fluorimetrically the release of free amine due to the hydrolytic activity of trypsin, either continuously or by measuring the amount of the released amine after a given time of incubation, the reaction being halted by addition of benzamidine in DMF. Figure 6 shows that the measured fluorescence intensity is strictly proportional to the concentration of trypsin in the range of 0–4 ng/ml (0–170 pmols). Conversely, N-benzoyl-arginyl-AEC was not hydrolyzed by a lysate of *L. polyphemus* amoebocytes even in the presence of 100 pg lipopolysaccharide. However, the alternative substrate,

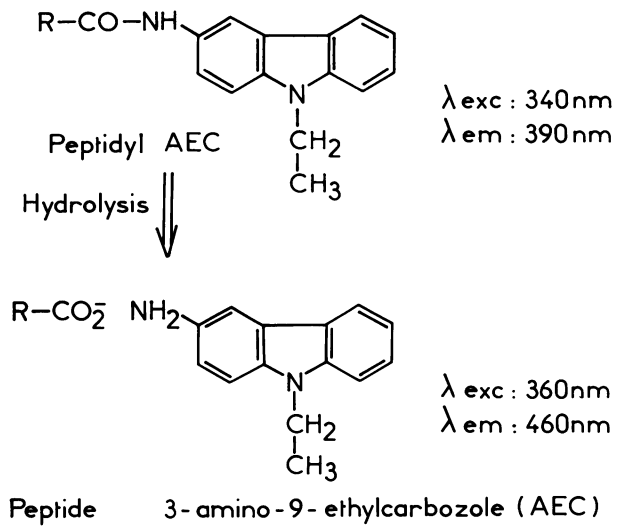

Fig. 1. Structure of an acyl-AEC and scheme of the hydrolysis reaction.

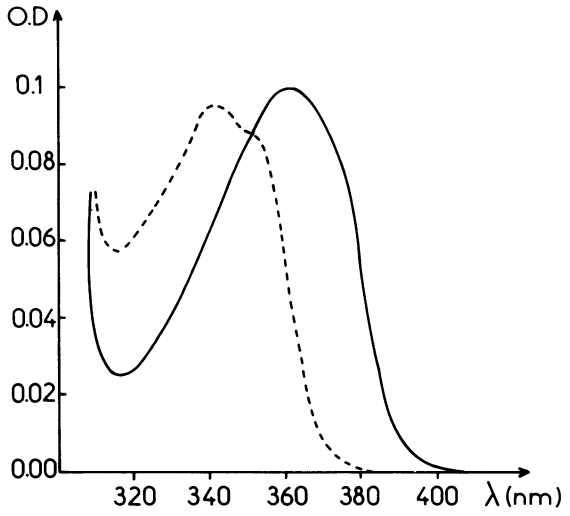

Fig. 2. Absorbance spectra of AEC (4.3 x 10^{-5} M) (———) and of N-benzoyl-arginyl-AEC (4.3 x 10^{-5} M) (------) in 0.1 M NaCl, 0.001 M $CaCl_2$, 0.05 M Tris-HCl buffer, pH 8.2.

tBOC-Val-Leu-Gly-Arg-AEC, was hydrolysed at a rate related to the lipopolysaccharide concentration (Figure 6). At 25°C, after an incubation time of 15 min, this method allowed us to detect as little as 1 pg lipopolysaccharide and to determine quantitatively the concentration of lipopolysaccharide in the range of 0–20 pg/ml. After 30 min incubation at 37°C, it is possible to detect the presence of <0.1 pg/ml (10^{-13} g/ml).

The following ways may be used to calculate the amount of released AEC enzyme or lipopolysaccharide from the enhancement of fluorescence: (i) the fluorescence spectrum of the substrate (tBOC-Val-Leu-Gly-Arg or N-benzoyl-arginyl-AEC) is recorded (λ excitation 352 nm) and the maximal fluorescence intensity at 460 nm (corresponding to a total release of the AEC) is calculated by multiplying the fluorescence intensity of the acyl derivates at 390 nm by 8.3; (ii) the maximal fluorescence intensity at 460 nm is determined after incubation of one of the substrates in the presence of 10 μg trypsin/ml for 30 min at 25°C. Under these conditions all the AEC is released from the substrate; (iii) the fluorescence intensity enhancement at 460 nm is recorded us-

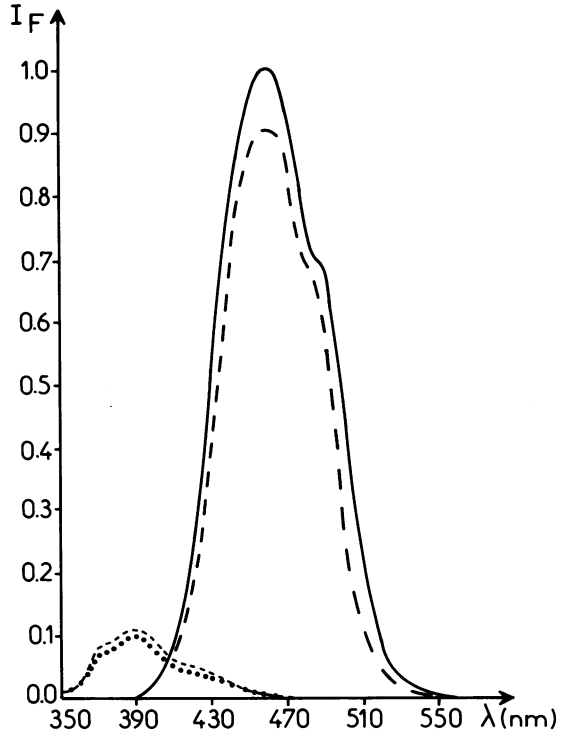

Fig. 3. Emission spectra of free AEC (2.1 x 10^{-5} M): excitation wavelengths: 360 nm (———) and 352 nm (– – –). Emission spectra of an acyl-AEC (2.1 x 10^{-5} M): excitation wavelength: 352 nm (·····) and 340 nm (------). All compounds were dissolved in 0.1 M NaCl, 0.05 M Tris-HCl buffer, pH 8.2. Temperature: 25°C.

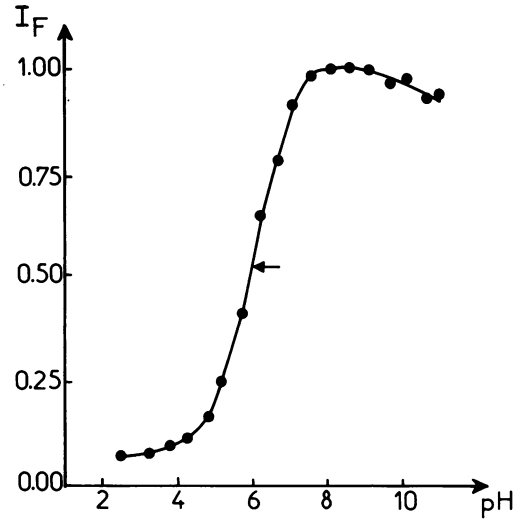

Fig. 4. Change of the fluorescence intensity of free AEC (2.4 x 10^{-5} M) related to the solution pH. (→) : pK of the excited state.

ing a solution of the substrate in the presence of a known amount of trypsin or of lipopolysaccharide and amoebocyte lysate.

Determination of the concentration of lipopolysaccharide in various samples

To determine accurately the concentration of lipopolysaccharide in various samples, the measurements of fluorescence intensity were recorded from the sample mixture and from four controls. The controls were used to avoid errors arising from contamination with: (i) hydrolytic enzymes in the sam-

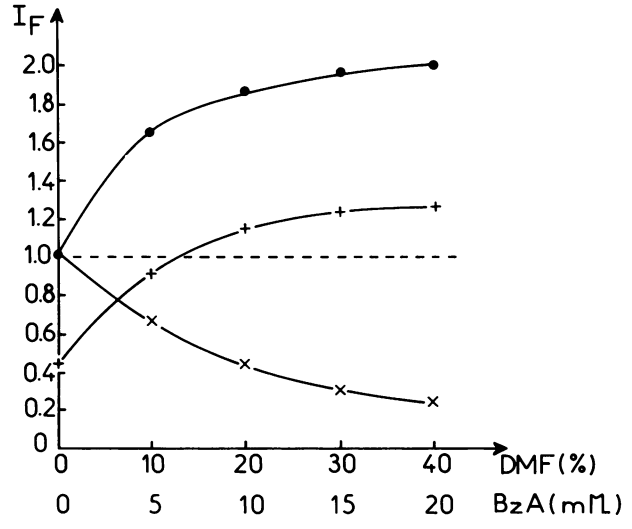

Fig. 5. Fluorescence intensity of AEC (2×10^{-5} M) as a function of DMF concentration (●——●), of benzamidinium chloride concentration (x——x) and of DMF concentration in the presence of 10 mM benzamidinium chloride (+ —— +). 0.01 M NaCl, 0.05 M sodium phosphate buffer, pH 7.1.

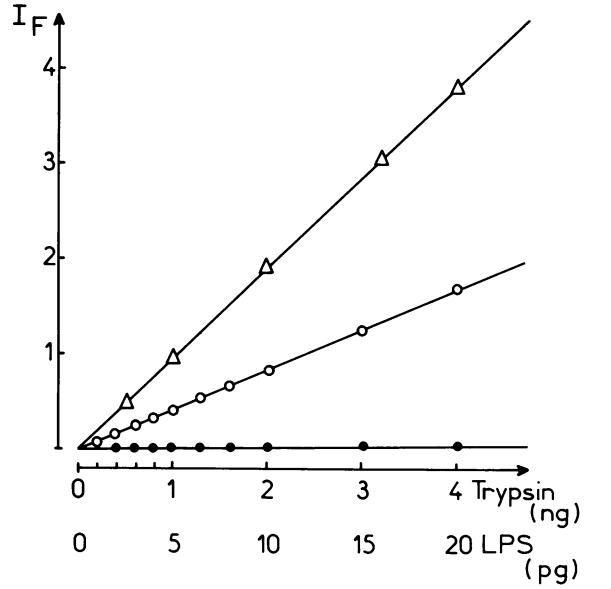

Fig. 6. Fluorescence intensity measured at 460 nm upon excitation at 352 nm of released AEC: (i) from 6×10^{-5} M tBOC-Val-Leu-Gly-Arg-AEC as a function of trypsin amount/test tube (△——△) and of lipopolysaccharide amount/test tube in the presence of *L. polyphemus* lysate (○ —— ○); (ii) from 4×10^{-5} M N-benzoyl-arginyl-AEC as a function of lipopolysaccharide amount/test tube in the presence of *L. polyphemus* lysate (● —— ●). 0.01 M NaCl, 0.05 M sodium phosphate buffer, pH 7.1, 25°C, 15 min. Enzymatic hydrolyses were stopped by addition of benzamidinium chloride in DMF.

ple; (ii) lipopolysaccharide in the buffer, in peptidyl-AEC solution and in test tubes; (iii) a quencher of AEC fluorescence; or (iv) an inhibitor of the *Limulus* test reaction. Furthermore, the sample solution should be diluted in a 10-fold series with a phosphate buffer to find the best conditions for accurate determination of lipopolysaccharide.

Double-distilled water using a quartz apparatus contains no lipopolysaccharide, unless the quartz apparatus had been used continuously for more than two months, when the distilled water was found to contain as much as 50 pg/ml lipopolysaccharide.

Various batches of commercial calf fetal serum were used to check the presence of lipopolysaccharides. It was found that calf fetal serum, in the absence of amoebocyte lysate, hydrolysed the peptide at a rate similar to that obtained with a solution containing 1 pg/ml lipopolysaccharide. However, in the presence of serum, lysate, and lipopolysaccharide, the rate of hydrolysis was ~ 80% that in the absence of serum.

Heparin at high concentrations (100 μg to 1 mg/ml) inhibited the hydrolysis of the substrate even in the presence of added lipopolysaccharide. However, at low concentration (10 μg/ml or less) no inhibition occurred.

Discussion

Lipopolysaccharides are made by gram-negative bacteria and may be present in any sterile solution. Lipopolysaccharides are known to induce a pyrogenic effect upon injection into animals and to elicit a large number of biological, and particularly immunological, effects (Morrison and Ryan, 1979). Furthermore, the detection of trace amounts of lipopolysaccharides could be relevant in diagnosis of a gram-negative septicemia. Detection of lipopolysaccharide is currently made by the determination of a pyrogenic effect after injection into rabbits; a time-consuming and very expensive procedure. Assay by gelation of *Limulus* amoebocyte lysates (Levin and Bang, 1964) has recently received attention, but this does not allow quantitative determination and cannot detect low concentrations of the order of a few picograms

With chromogenic substrates – which are specifically split by the lipopolysaccharide activated enzyme of the amoebocyte lysate – lipopolysaccharides may be determined at a level of 50–100 pg/ml (Nakamura *et al.*, 1977; Iwanaga *et al.*, 1978; Scully *et al.*, 1980; Thomas *et al.*, 1981). The use of an analogous fluorogenic substrate allows the determination of lipopolysaccharides at a concentration as low as 1 pg/ml. The fluorogenic compound AEC was selected for the following reasons: (1) the maximum excitation wavelength of AEC (360 nm) is outside the absorbance range of protein or nucleic acid chromophores and higher than that of other fluorophores such as methoxynaphthylamine (340 nm) or 7-amino-4-methyl-coumarin (345 nm) (Zimmerman *et al.*, 1976); (2) the fluorescence intensity is high, concentrations as low as 1 nM may be detected; and (3) at the maximum emission wavelength (460 nm) of AEC, the emission intensity of acyl-AEC is only 0.6% that of free AEC, upon excitation at the isosbestic point (352 nm).

The synthesis of the peptidyl-AEC is simple and yields are high. However, it should be noted that hydroxybenzotriazole is strictly required. Preliminary assays, using various solvents, such as pyridine, DMF, dimethylsulfoxide, or ethanol-water mixtures, and various activation methods, such as hydroxysuccinimide ester, dicyclohexylcarbodiimide, ethyl-1,2-dihydro-2-ethoxy-1-quinoline-carboxylate did not allow synthesis of any significant amount of acyl-AEC. The acyl-AEC is quite stable even in aqueous solution at neutral pH and at 25°C: no detectable amount of free AEC was released after 24 h.

The method described is quite sensitive; it is possible to detect <1 ng trypsin using N-benzoyl-Arg-AEC as substrate. Zimmerman *et al.* (1977), using a related coumarin substrate, were able to detect 50 ng trypsin. With t-BOC-Val-Leu-Gly-Arg-AEC as substrate, quantities as low as 1 pg lipopolysaccharide could be detected in 15 min. Thomas *et al.* (1981) used Bz-Ile-Glu-Gly-Arg-PNA as a chromogenic substrate to detect 10 pg/ml in 45 min.

The release of AEC can be monitored continuously as a function of time as long as the optimum pH of the protease activity is in the range 7 – 10 (Figure 4). The release of AEC can be monitored after a given incubation time by stopping the hydrolysis with a specific inhibitor. It is not possible to stop the reaction by addition of an acid such as acetic acid, because the fluorescence intensity in acidic medium is too low. However, with an inhibitor, such as benzamidinium chloride in the presence of DMF, proteases such as trypsin, plasmin, *L. polyphemus* proteases, or even elastase are quantitatively inhibited without any loss in the fluorescence intensity (Figure 5). Because *L. polyphemus* protease was not inhibited in the presence of 40% DMF, the addition of benzamidinium chloride was required. Other proteases, such as trypsin, are partially or totally inhibited in the presence of a large concentration of DMF. With such proteases, the inhibitor is not required to halt the hydrolytic process.

These studies clearly demonstrate the advantages of peptidyl-AECs as sensitive fluorogenic substrates for assays of proteases. Provided an appropriate peptide derivative is used, this fluorimetric method is the most sensitive described so far to determine the presence of trace amounts of lipopolysaccharide in biological systems and it represents an interesting tool in experimental biology and pathology. Several other peptide-AEC derivatives, which are specific substrates of enzymes such as elastase, plasmin, plasminogen activators, and cathepsins, have been synthesized and will be described elsewhere.

Materials and methods

L. polyphemus lysate (Malhindkrodt, USA) trypsin and fetal calf sera were from I.B.F. Réactifs (Villeneuve-La-Garenne, France); AEC was from Aldrich Europe (Beerse, Belgium) and was crystallized from a chloroform-hexane mixture. The peptide t-BOC-Val-Leu-Gly-Arg and N-benzoyl-arginine were purchased from Bachem (Bubendorf, Switzerland); *p*-toluene-sulfonic acid from Merck; 1-hydroxybenzotriazole and dicyclohexylcarbodiimide from Calbiochem.

Synthesis of acyl-AEC derivatives

The peptide t-BOC-Val-Leu-Gly-Arg (523.6 mg, 1 mmol) or N-benzoyl-arginine (278.31 mg, 1 mmol) were dissolved in freshly distilled DMF (2 ml) in the presence of p-toluene-sulfonic acid (172.20 mg, 1 mmol). The solution was kept at 0°C under nitrogen. To this solution, crystallized AEC (210.13 mg, 1 mmol), 1-hydroxybenzotriazole (119.16 mg, 1 mmol) and dicyclohexylcarbodimide (247.58 mg, 1.2 mmol) were successively added. The reaction mixture was kept in the dark at 0°C for 2 h and then at 25°C for 18 h. The reaction was monitored by t.l.c. on silicagel plates (Merck) using a chloroform/methanol/water mixture, 80:20:3 per volume; the peptide and derivatives were detected using Sakaguchi's reagent specific for the guanidium group. AEC and its acyl derivatives were detected under u.v. light as fluorescent compounds.

Dicyclohexylurea was precipitated by cooling the solution to 0°C for 1 h and removed by filtration. DMF was removed by evaporation under reduced pressure at 50°C. The acyl-AEC derivatives were purified by chromatography on a silicagel column (30 x 3 cm) equilibrated with a chloroform/methanol/water mixture, 80:20:3 per volume. The acyl-AEC derivatives were detected by their absorbance at 340 nm and their purity was checked by t.l.c. The fractions containing the expected compounds were concentrated under reduced pressure, redissolved in water, and freeze dried. (Yield: t-BOC-Val-Leu-Gly-Arg-AEC : 570 mg, 80%; N-benzoyl-arginyl-AEC: 400 mg, 85%).

Spectral characteristics of acyl-AEC derivatives

Absorbance spectra were recorded from a solution of AEC, t-BOC-Val-Leu-Gly-Arg-AEC or N-benzoyl-arginyl-AEC in 0.10 M NaCl, 0.05 M sodium phosphate buffer, pH 7.1 at 25°C, or in 0.1 M NaCl, 0.001 M $CaCl_2$, 0.05 M Tris-HCl buffer, pH 8.2 at 25°C. All samples were previously dried at 40°C under vacuum, for 48 h.

Fluorescence emission or excitation spectra were recorded using a FICA 55 spectrofluorimeter.

Solutions of AEC or acyl-AEC (4×10^{-5} M) were filtered through an HAPW 0.3 μm Millipore filter and poured into 5 x 5 mm cuvettes, at 25°C. The pH dependence of AEC fluorescence was determined using a stock solution of AEC (1.5 mg) in 150 μl of DMF diluted to 3 ml by adding water; the stock solution (100 μl) was added to 900 μl of the following buffers: a sodium citrate buffer for pH 2.6 – 7.05, Tris-HCl for pH 7.48 – 9.0, and sodium carbonate-bicarbonate for pH 9.59 – 10.80. Quantitative determinations of lipopolysaccharide were conducted in 0.1 M NaCl, 0.05 M sodium phosphate buffer, pH 7.1 with (6.0×10^{-5} M) tBOC-Val-Leu-Gly-Arg-AEC (excitation wavelength: 352 nm; emission wavelength: 460 nm) at 25°C or at 37°C.

Enzyme assays

Trypsin hydrolysis assays were performed in 0.001 M $CaCl_2$ - 0.1 M NaCl, 0.05 M Tris-HCl buffer, pH 8.2. A solution (0.6 ml) of the acyl-AEC (4×10^{-5} M) received various amounts of trypsin. After 15 min incubation, a solution (200 μl) of benzamidinium chloride (40 mM) in DMF was added to each sample. Alternatively, the rates of hydrolysis were monitored continuously by fluorescence measurement at 460 nm (excitation wavelength: 352 nm) without any addition of DMF containing benzamidinium chloride.

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