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The Synthesis and Analytical Use of a Highly Sensitive and Convenient Substrate of Elastase

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Elastase is thought to be the biochemical mediator of acute hemorrhagic pancreatitis (1) atherosclerosis (2) and lung emphysema (3). A clear understanding of its role in these diseases depends largely upon the availability of substrates which allow it to be determined in crude biological media or to be studied once isolated. For obvious reasons, such substrates should be sensitive, specific, sufficiently water-soluble and not subject to appreciable spontaneous hydrolysis. Besides, their enzymic degradation should be measurable by rapid and convenient means (e.g., spectrophotometric methods).

Natural elastase substrates like native or labeled elastin obviously do not meet these requirements (4). Although some of the model substrates which have been used in recent years to investigate the active center of elastase, are more sensitive and much more convenient than elastin, they do not satisfy all of the above requirements. For instance, BOC-alanine-*p*-nitrophenyl-ester described by Visser and Blout (5) is susceptible to trypsin and chymotrypsin, is poorly water-soluble and undergoes considerable spontaneous hydrolysis at pH 8.0. Acetyl-(alanine)$_3$-methyl-ester, proposed by Gertler and Hofmann (6) overcomes most of these disadvantages but its enzymic hydrolysis must be monitored by time-consuming potentiometric (6) or colorimetric (7) methods.

Peptide-*p*-nitroanilides whose breakdown may be monitored by a rapid spectrophotometric procedure, have been recently used in this laboratory for studying the subsite specificity of elastase (8). The most susceptible compounds of our series were acetyl-[4] and BOC-(alanine)$_3$-*p*-nitroanilide.

[3] Chargé de Recherche à l'I.N.S.E.R.M.

[4] Feinstein *et al.* (9) reported independently the action of elastase on this substrate and found a k_{cat}/Km value which is about 10 times higher than that reported by us.

The later substrate was stable at pH 8.0 in the absence of enzyme and could be used to assess very low elastase concentrations. However, its hydrophobic character rendered it poorly water-soluble and hence unsuitable for the separate determination of the kinetic constants k_{cat} and *Km*. For these reasons, we undertook the synthesis of a more soluble substrate in which the hydrophobic BOC group was replaced by a succinyl residue. The synthesis and analytical use of this compound are reported in this paper.

METHODS

Synthesis of Suc(Ala)$_3$-NA[5]

Melting points for all compounds were taken in capillary tubes and are not corrected. The purity of all compounds was checked by thin layer chromatography on Merck-Kieselgel 60 F_{254} ref. 5714. NMR and ir spectra are consistent with the expected structures. Optical rotations were taken with a Perkin–Elmer 141 polarimeter.

N-tert-Butoxycarbonyl-L-alanine p-nitrophenylester 1

Stir in an ice bath a mixture of 24.6 g (0.130 mole) of *N-tert*-butoxycarbonyl-L-alanine, 21.7 g (0.156 mole) of *p*-nitrophenol and 80 ml of ethyl acetate. While stirring is continued, add an ice-cold solution of 29.5 g (0.143 mole) of dicyclohexylcarbodiimide in 65 ml of ethyl acetate. The reaction mixture is then allowed to stand for 24 hr at room temperature. The white precipitate of dicyclohexylurea is separated by filtration and washed with small quantities of ethyl acetate. The washings are combined to the filtrate. After removal of the ethyl acetate under reduced pressure, a yellow oil remains which soon crystallizes after trituration with *n*-hexane; the solid is recrystallized from isopropyl-oxyde-*n*-hexane mixture (50:50) and gives *p*-nitrophenyl *N*-tert-butoxycarbonyl-L-alaninate **1** as shiny, tan, crystals, mp 126–127°. The yield is 27 g (67%).

Anal. Calcd for $C_{14}H_{18}N_2O_6$: C, 54.19; H, 5.85; N, 9.03. Found: C, 54.07; H, 5.94; N, 9.18.

N-tert-Butoxycarbonyl-L-alanyl-L-alanine-p-nitroanilide 2

Dissolve 21.4 g (0.087 mole) of L-alanine-*p*-nitroanilide hydrochloride in a mixture of 24.25 ml (0.174 mole) of triethylamine and 200 ml of dry methylene chloride. Remove the solvent and the excess of triethylamine under reduced pressure using a water-bath at 30–40°C. The residue is dissolved in methylene chloride and the solvent is again removed by

[5] Abbreviation used: Suc-(Ala)$_3$-NA: succinyl-L-alanyl-L-alanyl-L-alanine-*p*-nitro anilide.

evaporation. Then a solution of 27 g (0.087 mole) of *p*-nitrophenyl alaninate **1** in 200 ml of methylene chloride is added and the reaction mixture is allowed to stand overnight at room temperature. After this time the mixture is concentrated to dryness under reduced pressure and the crystallization is induced by treatment with isopropyl oxide. The crystals are collected and recrystallized in isopropyl alcohol to give *N-tert*-butoxycarbonyl-L-alanyl-L-alanine-*p*-nitroanilide **2** as cream, shiny, crystals, mp 198°, yield 30.5 g (92%).

Anal. Calcd for $C_{17}H_{24}N_4O_6$: C, 53.67; H, 6.36; N, 14.73. Found: C, 53.53; H, 6.36; N, 14.60.

L-alanyl-L-alanine-p-nitroanilide hydrochloride 3

Add 30.6 g (0.08 mole) of the *p*-nitroanilide **2** to a solution of 5.84 g (0.16 mole) of dry hydrogen chloride in 160 ml of acetic acid. The dissolution occurs with foaming and the hydrochloride precipitates rapidly.

Allow to stand 30 min at room temperature, add an equal volume of anhydrous ether and collect the hydrochloride on a sintered-glass funnel, wash the precipitate with anhydrous ether. Recrystallize by dissolution in methanol and progressive addition of ether. White needles of L-alanyl-L-alanine *p*-nitroanilide hydrochloride **3** are obtained: 19.6 g (77%) which do not melt but become charry; $[\alpha]_D^{20} = -58.2°$ (C = 1, H_2O).

Anal. Calcd for $C_{12}H_{17}ClN_4O_4$: C, 45.50; H, 5.41; N, 17.69. Found: C, 45.62; H, 5.41; N, 17.58.

N-tert-Butoxycarbonyl-L-alanyl-L-alanyl-L-alanine-p-nitroanilide 4

A suspension of 19.6 g (0.0618 mole) of L-alanyl-L-alanine-*p*-nitroanilide hydrochloride **3** in 880 ml methylene chloride and 35 ml triethylamine is stirred until complete dissolution. After this time, add 19.2 g (0.0618 mole) *N-tert*-butoxycarbonyl-L-alanine *p*-nitrophenylester **1** and allow to stand overnight at room temperature. Evaporate to dryness under reduced pressure, triturate the residue with isopropyl oxyde and collect the crystals on a sintered-glass funnel. Recrystallize in methanol and dry: 21 g (75%) of colorless crystals, mp 210° are obtained.

Anal. Calcd for $C_{20}H_{29}N_5O_7$: C, 53.20; H, 6.47; N, 15.51. Found: C, 53.04; H, 6.37; N, 15.69.

L-alanyl-L-alanyl-L-alanine-p-nitroanilide hydrochloride 5

Add 21 g (0.0465 mole) of the preceding BOC-tri-ala-*p*-nitroanilide **4** to a solution of 3.4 g (0.093 mole) of dry hydrogen chloride in 260 ml acetic acid. Warm the solution to 40°C and maintain this temperature until the dissolution is complete. Cool then to room temperature, add an

equal volume of anhydrous ether. The precipitate of tri-ala-*p*-nitroanilide hydrochloride is collected on a sintered-glass funnel and thoroughly washed with anhydrous ether. Recrystallize three times in absolute alcohol. L-Alanyl-L-alanyl-L-alanine-*p*-nitroanilide hydrochloride **5** is obtained as a white crystalline powder: 9.1 g (51%), mp = 206° $[\alpha]_D^{20} = -147.1°$ (C = 1, H_2O).

Anal. Calcd for $C_{15}H_{22}ClN_5O_5$, H_2O: C, 44.39; H, 5.96; N, 17.26. Found: C, 44.81; H, 5.99; N, 17.00.

*N-succinyl-L-alanyl-L-alanyl-L-alanine-p-nitroanilide (Suc-(Ala)$_3$-NA) **6***

Stir during four hours a mixture of 2.32 g (0.006 mole) of the $(Ala)_3$-NA hydrochloride **5**, of 250 ml chloroform and of 70 ml triethylamine. Evaporate to dryness under reduced pressure and without excessive heating ($t < 40°C$), dissolve the residue in chloroform and evaporate again. Then treat with 200 ml of chloroform and 1.2 g (0.012 mole) of succinic anhydride and stir the mixture overnight at room temperature. Evaporate to dryness, triturate the residue with water and collect the crystals. Recrystallize in 10% acetic acid in the presence of a small amount of "Norit" charcoal. Dry the tan crystals below 50° in the darkness, mp 246°; 1.8 g (67%) $[\alpha]_D^{19} = -24.8°$ (C = 1, DMF).

Anal. Calcd for $C_{19}H_{25}N_5O_8$: C, 50.55; H, 5.68; N, 15.52. Found: C, 50.61; H, 5.42; N, 15.44.

Substrate Dependency of the Initial Velocity (Fig. 1)

To study the influence of substrate concentration on the initial velocity in the absence of organic solvent (Fig. 1A), a 10 mM solution of Suc-$(Ala)_3$-NA was prepared in 0.2 M Tris buffer pH 8.0. The dissolution of the powder was complete after 15 min at room temperature and the pH was readjusted to 8.0 with 1 N NaOH. Appropriate dilutions of this stock solution were poured into 1 cm cuvettes and the enzymic reaction was started by adding a small aliquot of porcine elastase (Worthington Biochemical, lot ESFF, 2 LA) dissolved in the same Tris buffer. The release of *p*-nitroaniline was recorded for 2 min at 410 nm using a Zeiss PM QII spectrophotometer equipped with a thermostated cell holder (25°C).

When the rate was studied in the presence of N-methylpyrrolidone (Fig. 1B), a 250 mM substrate solution was prepared in this solvent by warming the suspension to 60°C for 10 min. The rates were measured as indicated above except that the enzymic reaction was started by adding small aliquots of appropriate dilutions of the 250 mM substrate solution to elastase dissolved in 0.2 M Tris buffer pH 8.0.

The overall rate constant k_{cat} was calculated from the maximal velocity by using ϵ = 8,800 M^{-1} cm^{-1} for *p*-nitroaniline at 410 nm (10) and 52,000 M^{-1} cm^{-1} for elastase at 280 nm (11).

Assay of Elastase (Fig. 2)

The following method may be used for testing the enzymatic activity of an elastase sample. A 125 mM stock solution of Suc-$(Ala)_3$-NA is prepared in *N*-methylpyrrolidone as indicated above. This solution is stable for months if stored at +4°C in a dark bottle. The sample to be tested for elastase activity is diluted with 0.2 M Tris buffer pH 8.0 and 2.5 ml of this dilution are poured into a 1 cm cuvette. After temperature equilibration at 25°C, the reaction is started with 20 μl of the stock solution of substrate and the optical density is recorded for 2–3 minutes at 410 nm. Modifications of this standard procedure as well as the detection of elastase on polyacrylamide gels will be described in the Results and Discussion section.

RESULTS AND DISCUSSION

The new substrate exhibits the classical absorption spectrum of *p*-nitroanilides (8, 10) with an absorbance maximum at 315 nm (ϵ = 14,600 M^{-1} cm^{-1}). It is much more water-soluble than the corresponding BOC or acetyl derivatives (8): its limiting solubility in 0.2 M Tris buffer pH 8.0 is about 20 mM (i.e., 17 *Km*).

As a consequence, k_{cat} and *Km* may be determined separately as shown in Fig. 1. In the absence of organic solvent (Fig. 1A), *Km* = 1.15 mM and k_{cat} = 16.6 sec^{-1}. It is worth mentioning that no activation by excess substrate was observed as in the case of trialanine-*p*-nitroanilide (8).

The rate of spontaneous substrate hydrolysis is extremely low: for a 1 mM solution it is 0.2 nM sec^{-1} at pH 8.0 and 25°C (0.07% substrate hydrolyzed per hr). This rate may thus be neglected when the enzymic reaction is measured within a few minutes. However, aqueous substrate solutions must be used within two days. To avoid this, it was found of interest to prepare stock solutions of Suc-$(Ala)_3$-NA in *N*-methylpyrrolidone in which the compound is stable for months. As can be seen from Fig. 1b, this solvent affects significantly the enzymic reaction although it is used at a concentration lower than 1%. Both constants are increased (*Km* = 2.4 mM and k_{cat} = 21.2 sec^{-1}) so that the overall susceptibility of the substrate is only slightly decreased. The unusually high sensitivity of elastase catalysis to organic solvents has already been reported (8, 12) and may be considered as another distinguishing feature of this protease. For BOC-alanine-*p*-nitrophenylester, *Km* = 0.3 mM and k_{cat} = 5.7 sec^{-1} at pH 6.5 (5) and for acetyl-(alanine)$_3$-methylester, *Km* = 0.43 mM and k_{cat} = 73 sec^{-1} at pH 8.0 (6).

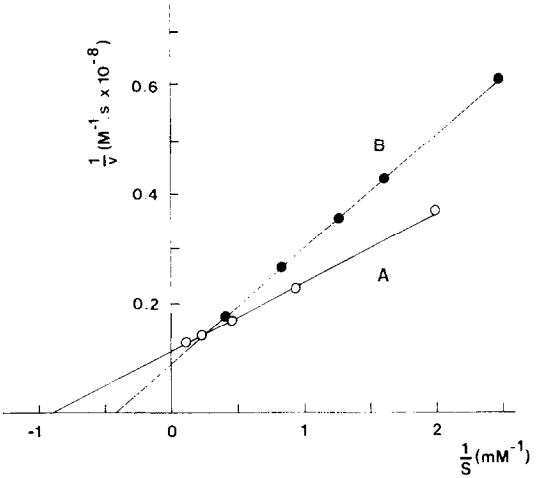

FIG. 1. Double reciprocal plot of the influence of substrate concentration on the initial rate in the absence of organic solvent (A) and in the presence of 0.92% (v/v) *N*-methylpyrrolidone (B). Conditions: 10 nM elastase, 0.2 M Tris–HCl pH 8.0, 25°C.

Figure 2 illustrates the high sensitivity and reliability of the new spectrophotometric method: elastase concentrations as low as 0.05 μg/ml (2 nM) may be assessed accurately within a few minutes. The assay is thus 20 times more sensitive than those using BOC-alanine-*p*-nitrophenyl-ester (5) or acetyl-(alanine)$_3$-methylester (6).

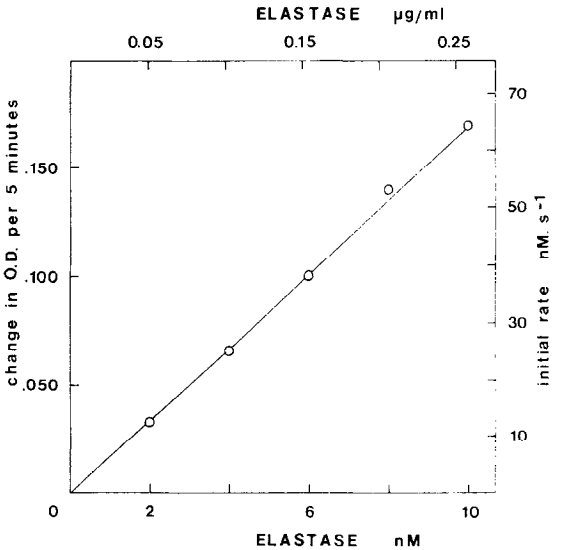

FIG. 2. Initial rate as a function of elastase concentration. Conditions: 1 mM Suc-(Ala)$_3$-NA, 0.2 M Tris–HCl pH 8.0, 0.8% (v/v) *N*-methylpyrrolidone, 25°C.

The two following modifications of the standard procedure outlined in the Methods section may be used either to increase the overall sensitivity or for the sake of technical convenience. First, the enzyme-substrate mixture may be incubated for longer periods (up to 1 hr) in a thermostated water-bath. The enzymic reaction is then stopped with 100 μl of glacial acetic acid which drops the pH to 3.7. The optical density at 410 nm is measured against a substrate control. This method is particularly suited for serial assays. Second, the liberated *p*-nitroaniline may be converted into a more coloured diazo-dye as described elsewhere (8, 13). Consequently, the overall sensitivity is increased by a factor of 5. This procedure is suited if the sample to be tested is yellow-colored (duodenal fluids or stool extracts).

When the substrate was tested against the parent enzymes trypsin and α-chymotrypsin (Worthington Biochemicals) by using the standard procedure, it was found that for identical enzyme concentrations the rate was 100 for elastase, 0.007 for trypsin and 0.014 for chymotrypsin. The assay appears thus to be considerably more specific than the method of Gertler and Hofmann using acetyl-(alanine)$_3$-methylester (6) for which the relative rates are 0.13 for trypsin and 1.2 for chymotrypsin.

Another interesting feature of Suc-(Ala)$_3$-NA is that it allows elastase to be detected on polyacrylamide gels. Fifty micrograms of elastase were electrophorized at pH 4.3 by the method of Reisfeld *et al.* (14); the gel was then immersed successively into 0.5 M Tris buffer pH 8.0 for 15 min and into a 1 mM substrate solution in 0.2 M Tris–buffer pH 8.0. After 10 min at 37°C, a yellow-colored band appeared on the gel, indicating the presence of elastase.

Suc-(Ala)$_3$-NA appears thus to be a very convenient analytical tool for solving medical or biochemical problems concerned with elastase.[6] Due to its chromogenic character, its high sensitivity and specificity and its small spontaneous hydrolysis, this substrate allows very low elastase concentrations to be measured in crude biological fluids or tissue extracts with an expeditious spectrophotometric method. Besides, it is sufficiently water-soluble to be used to investigate the active center of elastase under varying conditions or to measure inhibition constants.

SUMMARY

This paper describes the synthesis and analytical use of succinyl-(L-alanine)$_3$-*p*-nitroanilide, a new elastase substrate. At pH 8.0 and 25°C, the kinetic constants Km and k_{cat} are 1.15 mM and 16.6 sec^{-1}, respectively,

[6] The new substrate is now available from the Peptide Institute, 476 Ina, Minoh-Shi, Osaka, Japan.

in the absence of organic solvent and 2.4 mM and 21.2 sec^{-1} in the presence of 0.92% (v/v) *N*-methylpyrrolidone. An expeditious and highly sensitive spectrophotometric method for measuring elastase activity is described: enzyme concentrations as low as 0.05 μg/ml may be assessed accurately within a few minutes. The procedure may be used to assay elastase in crude biological material since the substrate is virtually not attacked by trypsin or chymotrypsin and does not undergo appreciable spontaneous hydrolysis. The new substrate may also be used to detect elastase on polyacrylamide gels.

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