BBA Report

BBA 61245

The use of *p*-nitrophenyl *N-tert*-butyloxycarbonyl-L-alaninate as substrate for elastase

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(Received March 7th, 1972)

SUMMARY

A sensitive, rapid spectrophotometric method for the determination of the esterase activity of elastase (EC 3.4.4.7) with the aid of a synthetic substrate, *p*-nitrophenyl *N-tert*-butyloxycarbonyl-L-alaninate, is described. At an assay pH of 6.5, $K_{m(app)} = 3 \cdot 10^{-4}$ M, $k_{cat} = 5.7\ s^{-1}$, and as little as 1 μg enzyme/ml can be satisfactorily determined.

Elastase (EC 3.4.4.7), otherwise known as pancreatopeptidase E, is a pancreatic protease unique among known mammalian enzymes in its ability to degrade elastin, an insoluble protein of yellow connective tissue[1]. Various assay methods for elastase are therefore based on its solubilization of elastin[2], but these procedures require relatively large amounts of enzyme and long assay periods. We reported earlier in a preliminary communication[3] that the protected amino acid ester, *p*-nitrophenyl *tert*-butyloxycarbonyl-L-alaninate (NBA: Cyclo Chemical Corp., Los Angeles, Calif.) is a good synthetic substrate for elastase. The conditions for this rapid spectrophotometric assay are presented below, and its usefulness is evaluated.

The basis for the assay is the release of *p*-nitrophenol from NBA by the esterase action of elastase, which is followed by measurement of the increase in absorbances at 347.5 nm. At this wavelength, the absorbance by *p*-nitrophenol ($\epsilon_{347.5\,nm} = 5.5 \cdot 10^3\ M^{-1} \cdot cm^{-1}$) is independent of pH[4], ensuring good reproducibility.

The standard assay conditions are as follows: To an exact volume (30–100 μl) of a 10^{-2} M stock solution of NBA in spectroquality acetonitrile or methanol in quartz

Abbreviation: NBA, *p*-nitrophenyl *N-tert*-butyloxycarbonyl-L-alaninate.

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reference and sample cuvettes (1-cm pathlengths) is added an appropriate amount of 0.05 M sodium phosphate buffer, pH 6.5. The absorbances are balanced at 347.5 nm, preferably in a double-beam recording spectrophotometer. 5 to 20 μg of elastase (twice crystallized porcine elastase (Worthington Biochemical Corp.) further purified by ion-exchange chromatography[5]) in a volume of 20 to 100 μl are added to the sample cuvette to a final volume of 3.0 ml. The increase in absorbance is followed for 2 to 3 min.

It was found that as little as 1 μg enzyme/ml reaction mixture could be satisfactorily assayed, and that the initial rate of NBA hydrolysis remained a linear function of elastase concentration up to a level of 14 μg enzyme/ml. The sensitivity of the NBA method thus compares favourably with the orceinelastin[6] and methyl furylacryloyl-L-alaninate[7] assays, in which upwards of 100 μg are used per activity determination.

A double reciprocal Lineweaver–Burk plot of the substrate concentration dependence gave a $K_{m(app)} = 3 \cdot 10^{-4}$ M and a catalytic rate constant ($k_{cat} = V/E_0$) of 5.7 s^{-1} for the hydrolysis of NBA at a pH of 6.5. The enzyme is specific for the L-isomer of NBA, since no reaction occurred with D-NBA. (Boc-D-alanine was prepared by the method of Schnabel[8] and converted, using dicyclohexylcarbodiimide, to D-NBA, m.p. 75–78 °C. $C_{14}H_{18}N_2O_6$ requiries: C, 54.19; H, 6.13; N, 9.03. Found: C, 54.14; H, 5.8; N, 9.04%.) The rate at which elastase is titrated by the active site titrant, diethyl *p*-nitrophenyl phosphate[9], was found to equal the rate of inhibition of enzyme activity towards NBA (Fig. 1).

The pH-rate (k_{cat}/K_m) profile in Fig. 2 for the hydrolysis of NBA by elastase is

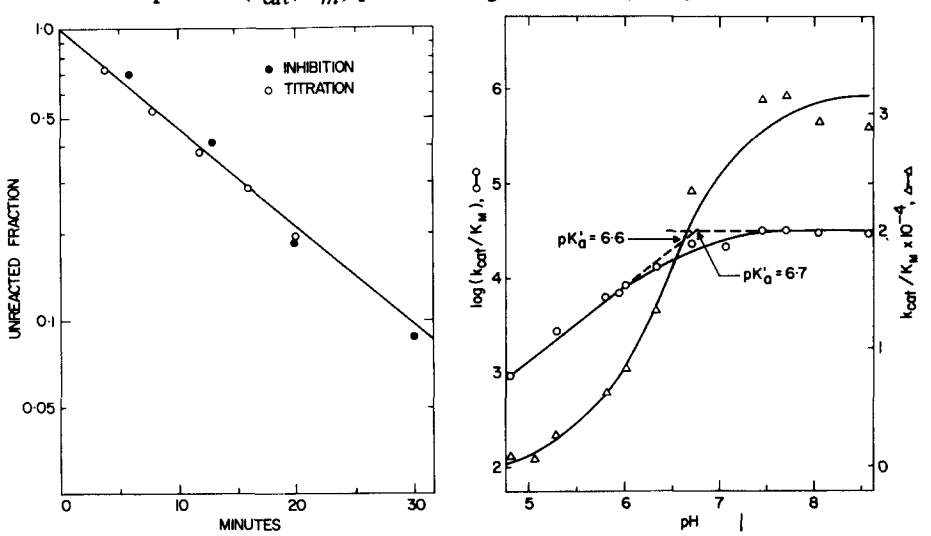

Fig. 1. Titration and inhibition rate of elastase by diethyl *p*-nitrophenyl phosphate with NBA as substrate. For titration, [diethyl *p*-nitrophenyl phosphate] = $4 \cdot 10^{-3}$ M in 4% methyl cyanide–0.08 M phosphate, pH 7.8; [elastase] = $1.54 \cdot 10^{-5}$ M. For inhibition, 25–100 μl of the titration mixture was assayed against $1 \cdot 10^{-4}$ M NBA at pH 6.5 in the standard manner.

Fig. 2. The rate of NBA hydrolysis by elastase as a function of pH. Acetate, phosphate, and carbonate buffers (0.05 M) were employed and assays were carried out with $1 \cdot 10^{-4}$ M NBA.

sigmoidal in shape. From the slopes of a log-rate–pH plot in the same figure, it would appear that only one ionizing group participates in catalysis[10]. This group has a pK_a' of about 6.7, suggesting that it is the imidazole side-chain of a histidine residue[11]. A histidine has also been implicated in the mechanism of action of elastase by crystallographic[12] and other solution[9] studies. An appreciable deuterium isotope effect became evident when the assay was carried out in ^2H_2O instead of H_2O as solvent ($k_{H_2O}/k^2_{H_2O} > 2$). If the transfer of a proton is the rate-limiting step in elastase catalysis, as has been suggested[9], a histidine could easily be involved by virtue of its general acid–base properties. The stereospecificity of the reaction, its pH dependence, and its inhibition by diethyl *p*-nitrophenyl phosphate confirm that NBA hydrolysis is a satisfactory assay for the enzyme.

The substrate NBA is also hydrolyzed by chymotrypsin (EC 3.4.4.5) at a pH of 6.5; the activity of chymotrypsin is, however, less than 10% of that of elastase. Trypsin (EC 3.4.4.4), as well as a number of other proteases and saccharases[13], does not act on this substrate at all. Thus, while the absolute specificity of the NBA assay does not appear to be as great as that of the spectrofluorimetric[14] or acetyltrialanyl methyl ester[15] assays, the short time and the low concentrations of a relatively simple substrate and the enzyme that are needed are features which could be exploited to advantage in many investigations.

During the development of the NBA assay, an investigation of the action of elastase on bacterial cell walls revealed that organisms with L-alanine residues in their peptide cross-links, *e.g. Micrococcus roseus*[16], are lysed by the enzyme, whereas others containing D-alanine residues, *e.g. Micrococcus lysodeikticus,* are not affected. Another finding related to the side-chain specificity of elastase was that the activity of the enzyme towards NBA was competitively inhibited ($K_i = 2 \cdot 10^{-3}$ M) in alkaline medium by the L-isomer but not the D-isomer of the antibiotic, cycloserine. Cycloserine can be regarded as a conformationally restrained alanine analogue, a fact that has been related to its antibacterial action[17] and which is ostensibly also responsible for its inhibitory effect on elastase.

We are pleased to acknowledge the support, in part, of this work by U.S. Public Health Service Grant AM-07300.

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