N-ACETYL-(L-ALA)$_3$-p-NITROANILIDE AS A NEW CHROMOGENIC SUBSTRATE FOR ELASTASE

Gad Feinstein, Abraham Kupfer and Mordechai Sokolovsky

Department of Biochemistry, Tel Aviv University,

Tel Aviv, Israel.

Received January 2, 1973

The introduction of a useful new chromogenic substrate for the determination of elastase (EC 3.4.4.7) activity is described. N-acetyl-L-Ala-L-Ala-L-Ala-p-nitroanilide (AcAla$_3$NA) is a new specific elastase substrate whose hydrolysis can be followed spectrophotometrically at 410 nm in a wide pH range. Its rate of hydrolysis by α-chymotrypsin (EC 3.4.4.5) and trypsin (EC 3.4.4.4.) is 0.02% and 0.001% respectively compared to its rate of hydrolysis by elastase. As little as 0.1 μg elastase/ml can be satisfactorily determined. At pH 8, K_m = 0.88 mM and k_{cat} = 11.9 sec^{-1}.

Pancreatic elastase is a proteolytic enzyme which is unique among the pancreatic enzymes in its ability to degrade elastin. The elastolytic activity of elastase is usually determined by using water-insoluble elastin as a substrate (1-4). Modified elastins, dye-bound, such as Congo red-elastin (5) or orcein-elastin (6) are also used as substrates for elastase. The elastolytic activity is followed by the rate of formation of water-soluble peptides, or dye-bound peptides, released from elastin by the enzyme. The fluoroscence of the solubilized peptides was used in several laboratories to follow elastase activity (7,8). Recently, the use of acid-solubilized elastin was reported (9). These assay methods are relatively lengthy and require large amounts of enzyme. It was also found that although trypsin and chymotrypsin, each by itself, do not degrade elastin, their presence in elastin-elastase reaction mixtures enhance the rate of elastin solubilization (10).

Abbreviations: AcAla$_3$NA, N-acetyl-L-Ala-L-Ala-L-Ala-p-nitroanilide; AcAlaOMe, N-acetyl-L-ala methyl ester; BzAlaOMe, N-benzoyl-L-Ala methyl ester; AcAla$_3$OMe, N-acetyl-L-Ala-L-Ala-L-Ala methyl ester; AcAla$_3$NH$_2$, N-acetyl-L-Ala-L-Ala-L-Alaninamide; BocAlaNP, N-tert-butyloxycarbonyl-L-Ala-p-nitrophenyl ester; ZAlaNP, N-carbobenzoxyl-L-Ala-p-nitrophenyl ester.

Subsequently, synthetic elastase substrates were introduced to facilitate faster determination of elastase activity and use of lower amounts of enzyme. The rate of hydrolysis of some of these substrates like BzAlaOMe (8) or $AcAla_3OMe$ (11) is followed titrimetrically. However, several micrograms of elastase are required for assay. The rate of BocAlaNP hydrolysis by elastase (12) can be followed spectrophotometrically. A distinct disadvantage of this substrate is that it is hydrolyzed by chymotrypsin and also undergoes considerable spontaneous hydrolysis at or above neutral pH.

We report here the introduction of a new, stable, chromogenic substrate, $AcAla_3NA$, which is highly specific for elastase. It can be used for a quick spectrophotometric assay of elastase by following the release of p-nitroaniline. As little as 0.1 µg elastase/ml can be easily determined and there is only negligible interference from other pancreatic proteolytic enzymes.

EXPERIMENTAL

Procine elastase prepared as described by Shotton (3) was a gift of Dr. A. Gertler. Bovine trypsin (Lot TRL 2DA) was purchased from Worthington Biochemical Corp. and bovine α-chymotrypsin (Lot 000079) from Calbiochem. Chicken ovoinhibitor was prepared as described (13). $AcAla_3NA$ was prepared for us by Miles-Yeda, Rehovot, Israel. All buffer reagents and organic solvents were of analytical grade.

Protein concentrations were determined spectrophotometrically using the following absorvancies ($A^{1\%}_{280}$) of 22.0 for elastase and 6.5 for ovoinhibitor. The following molecular weights were assumed: elastase, 25900 (14); ovoinhibitor, 46500 (13). Assay Method: Twenty mg of $AcAla_3NA$ were dissolved in ten ml of dimethylsulfoxide. Elastase was dissolved in 1×10^{-3}M acetic acid, diluted with the acid to the desired concentration, usually in the range of 1-20 µg/ml and kept in ice. The

enzyme, 0.1 ml, was added to a quartz cuevett containing 2.7 ml of 0.1 M Tris buffer, pH 8; the reaction was started by the addition of 0.2 ml of the $AcAla_3NA$ solution. The release of p-nitroaniline was followed at 410 nm (15) at 22°C, in a Varian double-beam spectrophotometer Model 635 equipped with a recorder. The Michaelis constant, K_m, was calculated from Lineweaver-Burk plot obtained with 0.05 - 0.30 mM substrate and 0.039 μM elastase. The pH-dependence of substrate hydrolysis was tested in the pH range 5.5 - 10.8. The following 0.1 M buffers were used: pH 5.5 - 7.0, phosphate; pH 7.5 - 8.5, Tris; pH 9 - 10.8, borate. The inhibition of elastase by ovoinhibitor was studied at pH 8. Ovoinhibitor was added to elastase in Tris buffer, mixed and allowed to stand for at least 10 minutes. The $AcAla_3NA$ was added and the residual elastase activity was then determined.

RESULTS AND DISCUSSION

$AcAla_3NA$ was found to be a highly specific substrate of elastase. At pH 8 it was hydrolyzed only very slowly by chymotrypsin or trypsin. The hydrolysis rates of $AcAla_3NA$ by chymotrypsin and trypsin, compared to elastase, were 0.02% and 0.001% respectively. The reported rates of hydrolysis of similar elastase substrates, e.g. $AcAla_3OMe$, by chymotrypsin and trypsin were 1.2% and 0.2% (11), while the rate of BocAlaNP hydrolysis by chymotrypsin was 10% (12).

The kinetic parameters of $AcAla_3NA$, together with those reported for other blocked alanine esters and amides as substrates of elastase, are compared in Table I. ZAlaNP (16) appears to be the best elastase substrate, as judged by its k_{cat}/K_m value. However, it was reported that both p-nitrophenyl esters, ZAlaNP and BocAlaNP, undergo fast spontaneous hydrolysis at neutral or above neutral pH. $AcAla_3NA$ was found to be stable in the pH range 5 - 10 with slow hydrolysis at pH 10.8. The sensitivity of ZAlaNP to chymotrypsin hydrolysis has not been reported.

TABLE I

Comparison of kinetic parameters of elastase substrates.

Substrate	pH	K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m (M^{-1} sec^{-1})	Reference
AcAlaOMe	8.0	153	6.7	43.7	11
BzAlaOMe	6.5	32	11.6	362	8
$AcAla_3OMe$	8.0	0.43	73	170000	11
$AcAla_3NH_2$	9.0	2.5	0.038	15	17
BocAlaNP	6.5	0.30	5.7	19000	12
ZAlaNP	7.9	0.60	110	185000	16
$AcAla_3NA$	8.0	0.88	11.9	13500	This work

AcAlaOMe is a poor substrate for elastase. Substituting the benzoyl for the N-acetyl blocking group gave a considerably better substrate, BzAlaOMe. The larger substrate, $AcAla_3OMe$, is even more sensitive to hydrolysis by elastase. The major change is observed in the Michaelis constant. K_m is lowered by a factor of about 100 while k_{cat} increases by a factor of 7. Thompson and Blout (17) compared a series of alanine methyl esters and found that $AcAla_4OMe$ was a better elastase substrate than $AcAla_3OMe$, solely due to the lower K_m values. Both these authors and Atlas et al. (18) suggested that the decrease in the value of K_m is due to increased interaction of the large substrates with sub-sites on the elastase molecule.

A comparison between $AcAla_3OMe$ and $AcAla_3NH_2$ reveals that the latter was less sensitive to elastase hydrolysis. However, in this case, the major difference was in k_{cat} (about 2000 fold). Similarly,

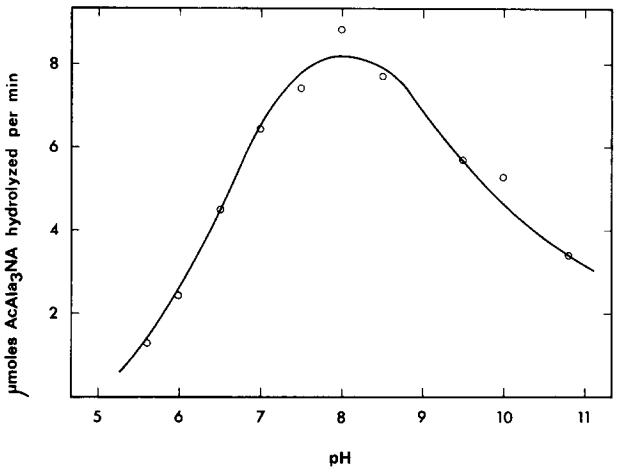

Fig. 1. The pH-dependence of hydrolysis of $AcAla_3NA$ (133 μg/ml) by elastase (1.0 μg/ml) at 22°C.

k_{cat} of $AcAla_4NH_2$ was much lower than that of $AcAla_4OMe$ (17). The K_m of $AcAla_3NA$ was quite close to that of $AcAla_3OMe$. The value of k_{cat} of the anilide was, on the other hand, only about one-tenth of that of the ester. This is probably due to the fact that in ester hydrolysis the deacylation is usually the rate limiting step, while in amide hydrolysis the acylation is the rate limiting step.

The pH-dependence of hydrolysis of $AcAla_3NA$ by elastase is shown in Fig. 1. The pH profile was bell-shaped, similar to results obtained with N-furylacryloyl-L-alanine methyl ester (16) and with $AcAla_3OMe$ (11). The pH optimum region appeared to be around pH 8 and it was narrower than with $AcAla_3OMe$.

The usefulness of $AcAla_3NA$ as a sensitive elastase substrate was further demonstrated by its use in following elastase inhibition as shown in Fig. 2. Ovoinhibitor was reported to be an elastase inhibitor (13). It was found in this study, using a relatively low concentration of enzyme, 0.5 μg/ml, that 0.95 mole of ovoinhibitor could inhibit completely one mole of elastase, in agreement with an earlier report (13).

The stability of $AcAla_3NA$ in a wide pH range, its high specifi-

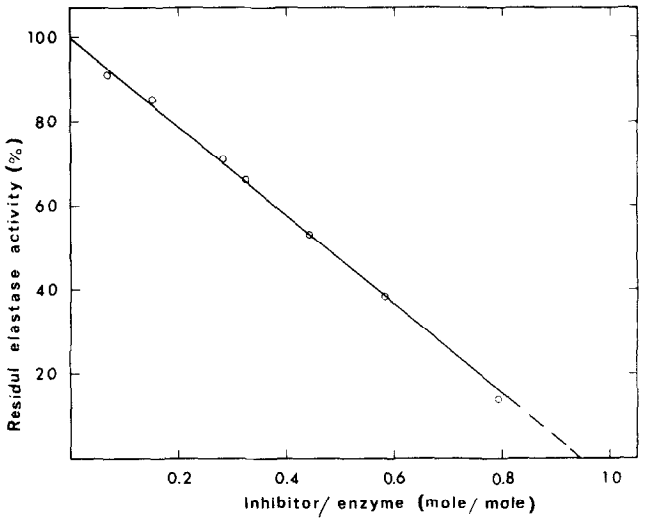

Fig. 2. The inhibition of elastase (0.5 μg/ml) by ovoinhibitor at pH 8, at 22°C.

city as a substrate of elastase in conjunction with a very slow rate of hydrolysis by other pancreatic enzymes, and the relatively high extinction coefficient of the released p-nitroaniline [ε_{410} = 8800 (15)], make it an excellent substrate suitable for an easy and quick spectrophotometric assay of elastase activity.

ACKNOWLEDGEMENT

This work was supported in part by a grant from the Israeli Ministry of Health.

REFERENCES

1. Partridge, S.M. and Davis, H.F., Biochem. J. 61, 21 (1955).
2. Mandel, I., Advan. Enzymol. 23, 1963 (1961).
3. Shotton, D.M., Methods Enzymol. 19, 113 (1970).
4. Hartley, B.S. and Shotton, D.M., The Enzymes, 3rd ed., p. 323 (P.D. Boyer - ed.) Academic Press, New York. 1971.
5. Naughton, M.A. and Sanger, F., Biochem. J. 78, 156 (1961).
6. Sachar, C., Winter, K.K., Sicher, R. and Frankel, S., Proc. Soc. Exp. Biol. Med. 90, 323 (1955).
7. Quinn, R.S. and Blout, E.R., Biochem. Biophys. Res. Commun. 40, 328 (1970).
8. Rinderknecht, H., Silverman, P. and Geokas, M.C., Enzymologia 40, 345 (1971).

9. Keller, S. and Mandel, I., Biochem. Med. 5, 342 (1971).
10. Gertler, A. and Birk, Y., Eur. J. Biochem. 12, 170 (1970).
11. Gertler, A. and Hofmann, T., Cand. J. Biochem. 48, 384 (1970).
12. Visser, L. and Blout, E.R., Biochim. Biophys. Acta 268, 257 (1972).
13. Gertler, A. and Feinstein, G., Eur. J. Biochem. 20, 547 (1971).
14. Shotton, D.M. and Hartley, B.S., Nature (London) 225, 802 (1970).
15. Erlanger, B.F., Kokowsky, N. and Cohen, W., Arch. Biochem. Biophys. 95, 271 (1961).
16. Geneste, P. and Bender, M.L., Proc. Nat'l. Acad. Sci. U.S. 64, 683 (1969).
17. Thompson, R.C. and Blout, E.R., Proc. Nat'l. Acad. Sci. U.S. 67, 1734 (1970).
18. Atlas, D., Levit S., Schechter, I. and Berger, A., FEBS Letters 11, 281 (1970).