Hoppe-Seyler's Z. Physiol. Chem.
Bd. 361, S. 1413–1416, September 1980

Synthesis and Analytical Use of 3-Carboxypropionyl-alanyl-alanyl-valine-4-nitroanilide: A Specific Substrate for Human Leukocyte Elastase

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(Received 8 June 1980)

Summary: A simple synthesis is described for 3-carboxypropionyl-Ala-Ala-Val-4-nitroanilide, a convenient and very specific substrate for human leukocyte elastase (K_m = 1.0mM, k_{cat} = 8.7 s^{-1}). The substrate does not undergo appreciable spontaneous hydrolysis. It is not cleaved by trypsin or chymotrypsin and only rather slowly by porcine pancreatic elastase (K_m = 9.1mM, k_{cat} = 1.4 s^{-1}).

Synthese und analytische Verwendung von 3-Carboxypropionyl-alanyl-alanyl-valin-4-nitroanilid: Ein spezifisches Substrat für Elastase aus menschlichen Leukozyten

Zusammenfassung: Eine einfache Synthese für 3-Carboxypropionyl-Ala-Ala-Val-4-nitroanilid wird beschrieben. Dieses Substrat ermöglicht eine bequeme und hochspezifische photometrische Bestimmung der Elastase aus menschlichen Leukozyten (K_m = 1.0mM, k_{cat} = 8.7 s^{-1}). Das Nitroanilid zeigt praktisch keine Spontanhydrolyse, wird von Trypsin oder Chymotrypsin nicht und von der Elastase aus Schweinepankreas nur sehr langsam (K_m = 9.1mM, k_{cat} = 1.4 s^{-1}) gespalten.

Key words: Elastase assay, leukocyte elastase, nitroanilide substrate, photometric assay, substrate synthesis.

4-Nitroanilides of small peptides have found broad application as synthetic substrates for various proteinases, because their hydrolysis is measurable by sensitive, rapid, and convenient means. (For a recent comparative study of different leaving groups in assays for serine proteinases see ref.[1]). Moreover, a high degree of specificity can be achieved by proper choice of the peptide sequence.

The most widely used peptide substrate for porcine pancreatic elastase has been Suc-$(Ala)_3$-Nan, which was introduced in 1974 by Kasafírek et

Enzymes:
Cathepsin G (EC 3.4.21.20); chymotrypsin (EC 3.4.21.1); elastase (EC 3.4.21.11); trypsin (EC 3.4.21.4).

Abbreviations:
Ac-, acetyl-; Boc-, *t*-butyloxycarbonyl-; Me_2SO, dimethylsulfoxide; Hepes, 2-[4-(2-hydroxyethyl)-l-piperazyl]-ethanesulfonic acid; MeOSuc-, methoxysuccinyl-; -Nan, -4-nitroanilide; -ONp, -4-nitrophenyl ester; Suc-, 3-carboxypropionyl-; Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulfonic acid; Z-, benzyloxycarbonyl-; mp, melting point.

0018-4888/80/0361-1413$02.00

al.[2] and Bieth et al.[3]. Recently the elastase from human leukocytes has attracted considerable interest, since it is thought to be involved in tissue destruction, which occurs for example in pulmonary emphysema[4]. Human leukocyte elastase has been routinely tested in several laboratories also using Suc-$(Ala)_3$-Nan. However, its k_{cat}/K_m ratio is only about 10% of the value for the porcine pancreas enzyme. This is not surprising, because it has been deduced both from the digestion of oxidized insulin B chain[5] and from inhibition studies with different chloromethyl ketones[6] that the human leukocyte enzyme prefers a valine residue in position P_1[7] rather than an alanine residue. Therefore, substituting Val for Ala at P_1 would be a promising approach to synthesize a more specific and sensitive substrate for human leukocyte elastase.

The corresponding compond Suc-Ala-Ala-Val-Nan was used as one of fourteen nitroanilides by Kasafírek et al.[8] in a study aimed at mapping the substrate binding sites of trypsin, chymotrypsin, and pancreatic elastase. However, no detailed description of the synthesis leading to the valine compound is given.

In this paper a straightforward and rather simple synthesis of Suc-Ala-Ala-Val-Nan is described together with its analytical use as a convenient substrate for human leukocyte elastase. The evaluated kinetic constants are compared with those of two more complicated tetrapeptide substrates which were introduced recently: Ac-Ala-Ala-Pro-Val-Nan by Zimmerman and Ashel[9] and MeOSuc-Ala-Ala-Pro-Val-Nan by Nakajima et al.[10].

Experimental

Enzymes

Porcine pancreatic elastase was purchased from Merck (Darmstadt, Germany).

Human leukocyte elastase was prepared essentially according to the method of Baugh and Travis[11].

Cathepsin G from human leukocytes was kindly provided by Bayer (Wuppertal, Germany).

Analytical procedures

Thin-layer chromatography was carried out on precoated silica gel 60 F_{254} plates from Merck (Darmstadt, Germany) in the system chloroform/methanol/acetic acid 85:10:5.

Elementary analyses gave satisfactory values of C, H, N for all compounds.

Amino acid analyses yielded the expected Ala/Val ratios within 7%.

Elastase assays

Stock solutions of substrates were prepared in Me_2SO. The assays were conducted at 25 °C in 0.1M Hepes buffer pH 7.5 containing 0.5M NaCl and 10% Me_2SO. The volume of all assays was 1 ml.

The increase in absorbance at 405 nm ($\epsilon = 9620 M^{-1} \times cm^{-1}$) due to 4-nitroaniline released was measured with an Eppendorf photometer 1101 M equipped with a calculator Eppendorf CKE 6453.

Kinetic constants

Values for K_m and k_{cat} were calculated from Lineweaver-Burk plots with substrate concentrations in the range 0.1–5.0mM. The concentrations for the elastases from porcine pancreas and human leukocytes were determined with Suc-$(Ala)_3$-Nan using published k_{cat} values[10], the value for leukocyte elastase being based on titrated enzyme.

Syntheses

Z-Val-Nan

To a solution of Z-Val (10.0 g, 39.8 mmol) and 4-nitroaniline (5.5 g, 39.8 mmol) in 80 ml tetrahydrofuran *N,N'*-dicyclohexylcarbodiimide (9.1 g, 44.1 mmol) in 55 ml of the same solvent was added. After stirring for 72 h the precipitated dicyclohexylurea was separated and washed with tetrahydrofuran. Evaporation of the combined filtrates in vacuo left a yellow solid, which was recrystallized from ethanol to yield 7.6 g (51%) of white crystals: mp. 178–181 °C; thin-layer chromatography: $R_F = 0.92$.

Val-Nan

To a suspension of Z-Val-Nan (6.3 g, 17.0 mmol) in 40 ml acetic acid, 40% HBr (26 ml) in the same solvent was added. After stirring for 2.5 h the clear solution was lyophilized. The residue was dissolved in methanol and the product precipitated by slow addition of dilute NH_4OH. A yield of tan crystals was obtained, 3.1 g (77%): mp. 99 °C; $R_F = 0.19$.

Boc-Ala-Val-Nan

A solution of Val-Nan (2.9 g, 12.2 mmol) and Boc-Ala-ONp (3.8 g, 12.2 mmol)[3] in 30 ml methylene chloride was stirred for 36 h, followed by removal of the solvent in vacuo. The residual oil was treated with dilute NH_4OH and crystallized from toluene as colorless needles. Yield 3.3 g (66%): mp. 169 °C; $R_F = 0.89$.

Ala-Val-Nan

Boc-Ala-Val-Nan (2.86 g, 7.0 mmol) was treated with a solution of HCl (0.91 g, 25.0 mmol) in 25 ml acetic acid for 1 h. Addition of 300 ml ether precipitated an oil, which solidified during prolonged stirring. It was filtered off, dissolved in methanol and the product precipitated upon slow addition of dilute NH_4OH. Recrystallization from toluene gave 1.84 g (85%) of colorless needles: mp. 192–193 °C dec.; $R_F = 0.11$.

Boc-Ala-Ala-Val-Nan

Ala-Val-Nan (1.80 g, 5.8 mmol) and Boc-Ala-ONp (1.81 g, 5.8 mmol)[3] were stirred in 85 ml methylene chloride. After 60 h 1.49 g of crystalline product could be collected by filtration. Concentration of the mother liquor to about 8 ml in vacuo and further stirring for 16 h led to precipitation of another 0.69 g product. Total yield 2.18 g (78%): mp. 110–111 °C dec.; $R_F = 0.85$.

Ala-Ala-Val-Nan

Boc-Ala-Ala-Val-Nan (1.95 g, 4.1 mmol) was treated with a solution of HCl (0.31 g, 8.5 mmol) in 8.5 ml acetic acid for 2 h. The yellow oil remaining after evaporation in vacuo was dissolved in methanol and the product precipitated by slow addition of NH_4OH. Recrystallization from toluene yielded 1.14 g (74%) of white needles: mp. 110 °C dec.; $R_F = 0.08$.

Suc-Ala-Ala-Val-Nan

A mixture of Ala-Ala-Val-Nan (0.50 g, 1.3 mmol) and succinic anhydride (0.50 g, 5.0 mmol) in 250 ml chloroform was stirred for 20 h, followed by evaporation of the solvent. After thorough trituration of the residue with 400 ml water for 5 h the product remained as white crystalline powder. Yield 0.51 g (81%): mp. 250 °C dec.; $R_F = 0.38$.

Results and Discussion

Starting from Val-Nan, the tripeptide substrate Suc-Ala-Ala-Val-Nan was synthesized by alternating acylations with Boc-Ala-ONp, Boc removal steps using HCl/acetic acid and final succinylation.

The substrate is readily soluble in Me_2SO, which is used as organic cosolvent in the assay system. The presence of Me_2SO is not a drawback in the case of human leukocyte elastase, on the contrary, the activity is increased by about 20% upon addition of 10% Me_2SO (v/v) (unpublished results). Spontaneous hydrolysis of Suc-Ala-Ala-Val-Nan can be neglected; a 1mM solution incubated for 24 h under assay conditions showed no increase in absorbance at 405 nm.

Neither trypsin nor chymotrypsin are able to cleave the substrate[8]. A rather slow hydrolysis was observed with a cathepsin G preparation which, however, was probably due to a minor contamination by human leukocyte elastase. Suc-Ala-Ala-Val-Nan is efficiently cleaved by human leukocyte elastase and very slowly by porcine pancreas elastase. The corresponding kinetic constants are presented in the table in comparison with the values for Suc-$(Ala)_3$-Nan and two

Table. Kinetic constants for the hydrolysis of 4-nitroanilide substrates by the elastases from human leukocytes (HL) and porcine pancreas (PP).

Substrate	Elastase source	K_m [mM]	k_{cat} [s^{-1}]	k_{cat}/K_m [$M^{-1} \times s^{-1}$]
Suc-$(Ala)_3$-Nan[a]	PP	5.9	37	6300
	HL	3.7	2.1	570
Suc-Ala-Ala-Val-Nan[c]	PP	9.1	1.4	150
	HL	1.0	8.7	8700
Ac-Ala-Ala-Pro-Val-Nan[b]	PP	13	22	1700
	HL	0.31	8.1	27000
MeOSuc-Ala-Ala-Pro-Val-Nan[a]	PP	6.2	17	2700
	HL	0.14	17	120000

[a] Data of Nakajima et al.[10]: 0.1M Hepes buffer, pH 7.5, 0.5M NaCl, 9.8% Me_2SO, 25 °C.
[b] Data of Zimmerman and Ashe[9]: 0.05M Tes buffer, pH 7.5, 10% Me_2SO, 25 °C.
[c] This work: 0.1M Hepes buffer, pH 7.5, 0.5M NaCl, 10% Me_2SO, 25 °C.

tetrapeptide substrates which were described recently[9,10].

Substituting Ala by Val in position P_1 of the most widely used elastase substrate Suc-$(Ala)_3$-Nan leads to a significantly better substrate for the human leukocyte enzyme in terms of both K_m and k_{cat}. The value of k_{cat}/K_m is increased by a factor of 15. This ratio is still higher for the tetrapeptide substrates, a fact mainly caused by very low Michaelis constants. At the same time the specificity is decreased. Both tetrapeptide nitroanilides are relatively good substrates for porcine pancreas elastase as well: with MeOSuc-Ala-Ala-Pro-Val-Nan k_{cat} is the same for the two elastases, with Ac-Ala-Ala-Pro-Val-Nan k_{cat} for the human leukocyte enzyme is even only one third of the value for the porcine pancreas enzyme. Suc-Ala-Ala-Val-Nan is the only substrate where k_{cat} is higher for human leukocyte than for porcine pancreas elastase, the factor being approximately 6.

Thus, because of its high specificity, its ease of preparation and the convenience of the spectrophotometric test, Suc-Ala-Ala-Val-Nan can be recommended for routine assays of the physiologically important human leukocyte elastase.

We are grateful to the *Stiftung Volkswagenwerk* for financial support. We are indebted to the *Deutsche Forschungsgemeinschaft* and the *Fonds der Chemischen Industrie* for kind supply of equipment.

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Notes added in proof (7 August 1980).
A further significant increase in sensitivity of the assay could be expected by using a fluorogenic leaving group, e.g. 7-amino-4-methylcoumarin[11]. Work with this intention is in progress.
The substrate will be available by Bachem AG, CH-4416 Bubendorf.

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