A NEW SUBSTRATE FOR ENTEROPEPTIDASE

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1. Introduction

Enteropeptidase (EC 3.4.4.8), a glycoprotein of mol. wt 200 000 is an enzyme of the small intestine [1–5]. Its physiological function is to catalyse the formation of trypsin from pancreatic trypsinogen, by the removal of a small amino-terminal peptide (trypsinogen activation peptide). The trypsin formed in this manner is essential for the activation of other pancreatic zymogens and the digestion of food proteins by cooperative enzymatic action [6]. Current assay methods for enteropeptidase activity [4,7–9] are based on the activation of trypsinogen and the subsequent splitting of an artificial substrate by trypsin. These methods are sensitive, but inherently complex, careful control of the assay conditions being necessary in order to avoid autoactivation and autodegradation of trypsinogen. In addition, the proteolytic action of trypsin upon the assayed material must be regarded as a serious drawback in subcellular localisation studies.

An assay method for enteropeptidase activity based on the hydrolysis of a specific artificial substrate would therefore have the double advantage of simplifying the assay procedure and removing uncertainties due to the generation of trypsin in the incubation mixture.

The amino acid sequence of activation peptides of trypsinogen from different species has been reported [10]. The partial sequence, –Asp–Asp–Asp–Asp–Lys has been shown by Maroux et al. [7] to comprise the substrate recognition and specificity sequence for enteropeptidase.

In this work we report the synthesis of tert-butyl–oxycarbonyl–tetra-L-aspartyl–L-lysyl–2-naphtylamide, a new substrate based on this sequence, and characterisation of its hydrolysis by enteropeptidase.

2. Materials and methods

2.1. *Abbreviations*

Z: Benzyloxycarbonyl–; Boc: tert-Butyloxycarbonyl; Bzl: Benzyl; –OSu: *N*-Succinimidoxy; TLC: thin-layer chromatography; solvent $CHCl_3/CH_3OH$ 9:1 (v/v); TFA: trifluoroacetic acid; NA: 2-naphthylamide.

2.2. *Materials*

Amino acid derivates, including N^{α}-Boc–N^{ϵ}-Z–L-lysine–2-naphthylamide were obtained from Bachem Feinchemikalien AG, Liestal, Switzerland. Porcine enteropeptidase was a gift of Opochimie, Monte Carlo, Monaco. Silicagel TLC plates (F_{254}) containing a fluorescent indicator, and other chemicals (reagent grade) were from Merck, Darmstadt, FRG.

2.3. *Peptide synthesis*

The protected substrate Boc–Asp(Bzl)–Asp(Bzl)–Asp(Bzl)–Asp(Bzl)–Lys(Z)–NA was prepared by the two-phase-method [11,12] by reacting N^{ϵ}-Z–L-lysine–NA stepwise and successively with four Boc–

Table 1
Yields and characterisations of intermediary peptides

Peptide		Reaction		Yield		TLC[b]	Amino acid analysis
	mol. wt	Molar ratio[a]	Hours	mmoles	%	R_F	Asp/Lys
Boc–Asp(Bzl)–Lys(Z)–NA	710.80	1:10	20	1.86	93	0.72	1.00:1.00
Boc–[Asp(Bzl)]$_2$–Lys(Z)–NA	916.01	1:11	23	1.75	94	0.74	2.01:0.99
Boc–[Asp(Bzl)]$_3$–Lys(Z)–NA	1121.22	1:10	23	1.65	94	0.75	3.03:0.97
Boc–[Asp(Bzl)]$_4$–Lys(Z)–NA	1326.43	1:17	21	1.54	94	0.76	3.99:1.01

[a]Molar ratio of reactants: protected aspartic acid hydroxysuccinimide ester/N-terminal-deprotected peptide.
[b]TLC: Thin-layer chromatography on silica-gel coated plates. Solvent system chloroform/methanol 9:1 (v/v).

Asp(Bzl)–OSu, the N-terminal Boc-protection being removed from the intermediary peptides with cold trifluoroacetic acid according to standard procedures. The elongations were performed in CH_2Cl_2. The progress of the reactions was followed by TLC, substance spots being detected by ultraviolet light (254 nm), I_2-vapour, ninhydrin and fluorescamine reagents. After virtually complete reaction had been established by the absence of amino-peptide stainable with ninhydrin the two-phase extraction with 0.5 M citric acid, H_2O, 0.3 M K_2CO_3 and H_2O. Further characteristics of the synthesis are summarized in table 1.

2.4 *Enteropeptidase solution*

Freeze-dried porcine enteropeptidase was dissolved in 10 mM Tris acetic acid buffer, pH 6.0 and subjected to gel permeation chromatography on Sephadex G-100. Fractions containing enteropeptidase and aminopeptidase activity, eluting just after the void volume, and well removed from fractions containing trypsin activity, were pooled. The pooled fractions were concentrated by negative pressure dialysis and dialysed against distilled water. All operations were carried out at 0–5°C.

2.5. *pH optimum*

In the following order were mixed (a) 0.25 ml of 1 mM substrate (tert-butyloxycarbonyl–tetra-L-aspartyl–L-lysyl–2-naphthylamide) dissolved in 50% aq. methanol. (b) 0.5 ml of Tris-(hydroxymethyl)-aminomethane/HCl buffer, 50 mM, containing 2 mM $CaCl_2$ and the calculated quantity of NaCl to produce an ionic strength of 0.05. (c) 0.25 ml of enteropeptidase solution prepared as in 2.4. Duplicate samples were prepared containing water in place of enzyme solution to allow for autolysis of substrate. The solutions were incubated 4 h at 37°C, and the enzymic hydrolysis inhibited by the addition of 0.50 ml of 40% aq. trichloroacetic acid. The liberated 2-naphthylamine was measured by the colorimetric method of Goldbarg and Rutenburg [13].

2.6. *Determination of Michaelis constant*

The determination was carried out in the same buffer as the pH optimum (7.9) found in 2.6. Substrate concentration range was 0.1–0.5 mM, and time of incubation 0–5 h. Initial rates of hydrolysis of the substrate were determined by graphical methods, and the resultant data treated by the methods of Lineweaver and Burk [14].

3. Results and discussion

The two-phase-method synthesis of peptides requires no elaborate or expensive equipment and produced excellent yields of intermediary peptides and protected substrate, in spite of the size and hydrophobic nature of the benzyl ester protecting groups employed. Due to the highly hydrophobic nature of the growing protected peptide, it was found impracticable to remove significant quantities of amino-peptides with the liquid–liquid extractor. However, the employment of a 10–20% molar excess of active ester was sufficient to bring the reaction to virtual completion and to obviate the requirement for special purification techniques. Elemental analysis of

the protected substrate, Boc–[Asp(Bzl)]$_4$–Lys(Z)–NA, $C_{73}H_{79}N_7O_{17}$ mol. wt 1326.43 gave the following: %C 66.04 (Calc. 66.10), %H 6.09 (Calc. 6.00). %N 7.51 (Calc. 7.39).

Hydrogenolysis of the protected substrate (0.50 g, 0.38 mmol) in aqueous HOAc with palladium/carbon catalyst in a hydrogenation apparatus, gave the substrate, (0.30, 0.37 mmol) Boc–(Asp)$_4$–Lys–NA, $C_{37}H_{49}N_7O_{15}$, mol. wt 831.82 as a bulky white powder after filtration and lyophilisation. Amino acid ratio: Asp/Lys 4.02:0.98.

Selective removal of the Bzl– and Z– protecting groups by hydrogenolysis results in a substrate in which the terminal amino group remains protected with a Boc group. This simulates the continuing peptide chain in the natural substrate, while protecting the substrate against possible attack by aminopeptidase, a contaminant of enteropeptidase preparation.

Incubation of the substrate with enteropeptidase in aqueous solution resulted in the liberation of 2-naphthylamine. Figure 1 shows the pH optimum curve for the enteropeptidase-catalysed hydrolysis of the substrate. Of particular interest is the shoulder of activity extending down to pH 5.5. In assay methods using trypsinogen as substrate, the initial activation process is performed at pH 5.6, in order to minimise autocatalysis, which becomes significant with increasing pH. Although we cannot, therefore, directly compare the pH optimum curve determined here with the apparent pH optimum curve for the hydrolysis of trypsinogen, we believe that the two would be very similar if corrections for autocatalysis could be applied to the latter. We infer that the same pH dependence of hydrolysis rate applies to the new artificial substrate as to the natural substrate.

As shown in fig.2, Michaelis-Menten kinetics apply to the hydrolysis of the substrate by enteropeptidase. This has also been shown to be the case for the natural substrate. It is invalid to compare the value of 0.32 mM found for K_m with the value found by Maroux et al. [7], since the pH and other conditions are different in the two cases. It may nevertheless be regarded as typical for serine protease/artificial substrate systems [15,16].

Methods based upon the generation of trypsin in the incubation mixture necessarily include the possibility of the proteolytic release of bound enteropeptidase, when applied to fractionated subcellular material. The new substrate does not produce this undesirable feature of previous assays and will therefore find use in such studies.

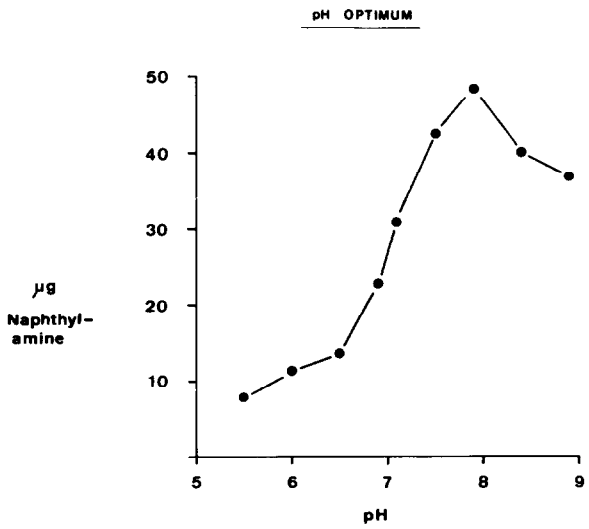

Fig.1. Determination of the pH optimum for the enteropeptidase-catalysed hydrolysis of Boc–[Asp]$_4$–Lys–2-naphthylamide. See methods for conditions of determination.

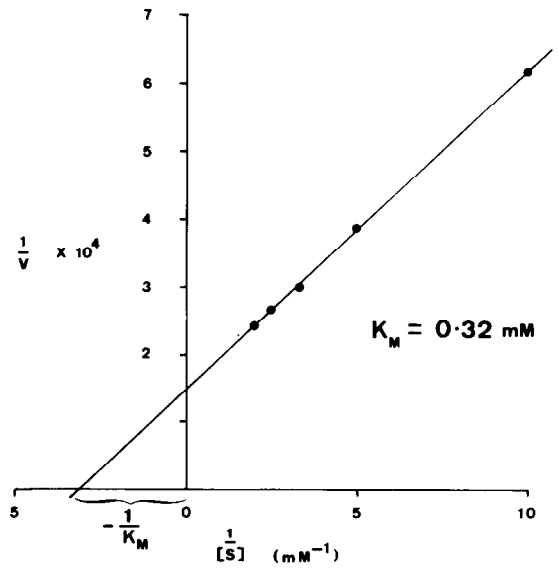

Fig.2. Determination of the Michaelis constant, K_m, for the enteropeptidase-catalysed hydrolysis of Boc–[Asp]$_4$–Lys–2-naphthylamide at the pH optimum 7.9 (Lineweaver–Burk plot). Other conditions under Materials and methods.

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