Specificity studies on enteropeptidase substrates related to the N-terminus of trypsinogen

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The specificity of the synthetic substrate Gly-[L-Asp]₄-L-Lys 2-naphthylamide, originally developed for the assay of enteropeptidase (EC 3.4.21.9), was investigated with partially purified aminopeptidase. Our results indicate that, not only enteropeptidase, but also the concerted action of the aminopeptidases of the rat small intestine, can rapidly release 2-naphthylamine from the substrate. A previously undescribed, highly active, dipeptidylaminopeptidase, which hydrolyses a Gly-Asp dipeptide from the N-terminus of the substrate, was detected in rat small intestine. The resulting [L-Asp]₃-L-Lys 2-naphthylamide fragment is then degraded by a combination of aminopeptidase A and N to yield free 2-naphthylamine. Thus the present substrate cannot be regarded as being specific for enteropeptidase, and its use leads to an over-estimation of enteropeptidase activity in homogenates and extracts of intestinal tissue. In order to prevent this non-specific hydrolysis by aminopeptidases, stereoisomeric substrates with the sequence L-Ala-D-Asp-[L-Asp]₃-L-Lys methyl ester, D-Ala-[L-Asp]₄-L-Lys methyl ester and L-Ala-[Asp]₄-L-Lys methyl ester were synthesized and tested as alternative substrates by their ability to inhibit the enteropeptidase-catalysed activation of trypsinogen.

INTRODUCTION

Enteropeptidase (EC 3.4.21.9) initiates the activation of pancreatic zymogens in the small-intestinal lumen by converting trypsinogen into trypsin, which in turn activates all other pancreatic zymogens. The conversion of trypsinogen into trypsin is achieved by hydrolysis of the Lys₍₆₎-Ile₍₇₎ bond (Yamashina, 1956), with the subsequent release of an activation peptide, containing an unusual tetra-aspartic acid sequence, which has been shown to play an important role in the recognition and activation of trypsinogen by enteropeptidase (Maroux *et al.*, 1971).

Until recently, the assay systems for enteropeptidase were based on the two-step procedure as described originally by Kunitz (1939*a,b*) in which trypsinogen is first incubated with a solution containing enteropeptidase and then, in a second step, the trypsin generated is determined with α -N-benzoyl *p*-nitroanilide. The main difficulty lies in the prevention of trypsinogen auto-activation: the zymogen has to be activated at low pH values far away from the pH optimum for enteropeptidase, thereby considerably diminishing the sensitivity of the assay.

Hesford *et al.* (1976) described a new one-step assay with the synthetic substrate α -Boc-[L-Asp]₄-L-Lys-Nap, later modified by Antonowicz *et al.* (1980) to Gly-[L-Asp]₄-L-Lys-Nap, in which the Lys-Nap bond is hydrolysed by enteropeptidase to liberate 2-naphthylamine, which can be measured fluorimetrically. Use of this substrate has the advantage over previous methods that the effects of changes in such parameters as ionic strength, pH or activation by ions can be monitored directly.

However, during the chromatographic purification of rat and human enteropeptidase, especially by affinity

chromatography, we detected several peaks of enzymic activity. As it was considered unlikely that all contained enteropeptidase, a detailed study was undertaken to investigate possible interference by aminopeptidase and to improve the specificity of the substrate. A preliminary account of this work has been presented (Jenö *et al.*, 1984).

EXPERIMENTAL

Materials

The synthetic substrate Gly-[L-Asp]₄-L-Lys-Nap, the protected tripeptide α -Boc-[L-Asp(β -Bzl)]₂-L-Lys-(N⁶-Cbz)-Nap and the protected amino acids α -Boc-L-Asp(β -Bzl)-OSu, α -Boc-D-Asp(β -Bzl), N⁶-Cbz-L-Lys-OMe, α -Boc-L-Ala-OSu, α -Boc-D-Ala and the aminopeptidase substrates α -L-Glu-Nap and L-Ala-Nap were from Bachem (Bubendorf, Switzerland). Crystallized bovine trypsinogen (type I) was from Sigma Chemical Co. (Poole, Dorset, U.K.). CNBr-activated Sepharose 4B, DEAE-Sephadex A-25 and Sephadex G-10 were from Pharmacia (Uppsala, Sweden), and Ultrogel AcA 34 was obtained from LKB (Bromma, Sweden). Acetonitrile for h.p.l.c. was from Fluka (Buchs, Switzerland).

Methods

Substitution of CNBr-activated Sepharose 4B. For affinity chromatography, CNBr-activated Sepharose was substituted as described by Grant *et al.* (1978) except that the spacer arm (glycylglycine) was coupled at a concentration of 0.1 mmol/g dry wt. of Sepharose. The gel was stored in 1 mM-HCl until use.

Preparation of enteropeptidase. Adult female rats were kept without food overnight and then anaesthetized with diethyl ether. The first 20 cm of the small intestine was

Abbreviations used: Boc, t-butoxycarbonyl; Cbz, benzyloxycarbonyl; Bzl, benzyl; Nap, 2-naphthylamide; OSu, hydroxysuccinimide; OMe, methyl ester; Dns, dansyl (5-dimethylaminonaphthalene-1-sulphonyl).

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Fig. 1. Elution profile from an Ultrogel AcA 34 column of brush-border membrane hydrolases solubilized in Triton X-100

●—●, Enteropeptidase; ▲—▲, aminopeptidase A (10 $\times$); ○—○, aminopeptidase N. The bar indicates the fractions containing enteropeptidase that were pooled for specificity studies with the synthetic substrate or further purified by affinity chromatography.

removed and rinsed with ice-cold 0.15 M-NaCl and cut lengthwise to expose the mucosa. The mucosa was scraped from the everted small intestine with a glass slide and taken up in 4 vol. of 50 mM-Tris/HCl buffer, pH 8, containing 0.1% (v/v) Triton X-100 (buffer A), homogenized in an Ultra-Turrax homogenizer and stirred for 1 h at 4°C. The homogenate was spun at 6000 g for 30 min and the resulting supernatant centrifuged at 105000 g for 60 min. A 5 ml portion of the high-speed supernatant was applied to a column packed with Ultrogel AcA 34 (2.5 cm $\times$ 90 cm) and developed with buffer A at a flow rate of 20 ml/h (see Fig. 1). The fractions containing enteropeptidase were pooled and stored at 4°C.

In order to separate enteropeptidase from aminopeptidase, the pooled enteropeptidase fractions from the Ultrogel AcA 34 column were pumped on to a column packed with *p*-aminobenzamidine-substituted Sepharose (1.5 cm $\times$ 10 cm) (Grant *et al.*, 1978). The column was washed with buffer A until all the unbound peptidase activity was eluted (see Fig. 2). Enteropeptidase was desorbed with 50 mM-Tris/50 mM-maleate buffer, pH 5.6, containing 0.1% (v/v) Triton X-100, then dialysed against buffer A and stored at 4°C until use.

Enzyme assays. Aminopeptidases A and N were determined fluorimetrically with α -L-Glu-Nap and L-Ala-Nap as described by Sterchi & Woodley (1980). Enteropeptidase was measured fluorimetrically with Gly-[L-Asp]₄-L-Lys-Nap as described by Antonowicz *et al.* (1980) or with trypsinogen as substrate as described by Hadorn *et al.* (1969).

Separation of the breakdown products of Gly-[L-Asp]₄-L-Lys-Nap. The digestion of the synthetic substrate was monitored by reversed-phase h.p.l.c. Peptide substrate (130 nmol) was digested either with the crude entero-

Fig. 2. Separation of aminopeptidases and enteropeptidase on *p*-aminobenzamidine-substituted Sepharose

Right-hand scale: ●—●, enteropeptidase, determined with trypsinogen as substrate. Left-hand scale: ○—○, aminopeptidase N; ▲—▲, aminopeptidase A (10 $\times$); ●····●, liberation of 2-naphthylamine from the synthetic substrate Gly-[L-Asp]₄-L-Lys-Nap. The arrow indicates desorption of enteropeptidase with Tris/maleate buffer.

peptidase pool from Fig. 1 or with the unbound aminopeptidase fraction from the affinity column (see Fig. 2) in a final volume of 200 μ l at 37 °C. After 1 h the sample was frozen and freeze-dried. Protein in the freeze-dried material was precipitated with 70% (v/v) methanol and removed by centrifugation at 10000 g for 5 min. The supernatant was dried under a stream of N_2 and taken up in a small volume of 155 mM-KCl, pH 2.2, containing 25% (v/v) acetonitrile. The peptide fragments were separated on a Beckman 5 μ m-particle-size Ultrasphere ODS reversed-phase column by using a gradient of 30–50% (v/v) acetonitrile in 155 mM-KCl, pH 2.2, at a flow rate of 1 ml/min. The peak areas were quantified with a Hewlett-Packard 3380 A integrator.

Kinetic assays. Bovine trypsinogen was activated by the method of Hadorn *et al.* (1969) with rat enteropeptidase that had been purified by affinity chromatography (enteropeptidase pool from Fig. 2; specific activity 109 nmol of trypsin/min per mg of protein). The concentration of trypsinogen was varied between 15 and 50 μ M. For inhibition assays, the synthetic peptides were dissolved in water and added to the activation mixture to give a final concentration of between 20 and 50 μ M.

For the determination of K_m and initial velocities, M_r values of 24000 for trypsinogen and 23500 for trypsin were assumed. The data were plotted in accordance with Lineweaver & Burk (1934) and the parameters V_{max} , K_m and K_i determined by weighted regression analysis (Cornish-Bowden & Endrenyi, 1981).

Peptide synthesis. The hexapeptides L-Ala-D-Asp-[L-Asp]₃-L-Lys-OMe (peptide I), D-Ala-[L-Asp]₄-L-Lys-OMe (peptide II) and L-Ala-[L-Asp]₄-L-Lys-OMe (peptide III) were synthesized in solution starting from α -Boc-L-Lys(N^{ϵ} -Cbz)-OMe by stepwise elongation with α -Boc-L-Asp(β -Bzl)-OSu according to standard procedures (Hesford *et al.*, 1976). The D isomers were coupled by the dicyclohexylcarbodi-imide/1-hydroxybenzotriazole procedure (König & Geiger, 1970a,b,c). [L-Asp]₂-L-Lys-Nap and [L-Asp]₃-L-Lys-Nap were synthesized from α -Boc-[L-Asp(β -Bzl)]₂-L-Lys(N^{ϵ} -Cbz)-Nap. Final deprotection of all peptides was achieved by first removing the t-butoxycarbonyl group in 70% (v/v) trifluoroacetic acid (Schnabel *et al.*, 1971), followed by catalytic hydrogenation in acetic acid to remove the benzyl and benzyloxy-carbonyl groups. The peptides were purified by gel filtration on Sephadex G-10 in 50 mM-ammonium acetate, pH 5.6, followed by ion-exchange chromatography on DEAE-Sephadex A-25 with a linear gradient of 10 mM–0.6 M- NH_4HCO_3 , pH 7.5. No detectable impurities were found in the peptides by t.l.c. on Silica-gel 60 F-254 plates developed with ethanol/water (7:3, v/v) or by high-voltage paper electrophoresis with pyridine/acetic acid/water (25:1:225, by vol.), pH 6.5 (Magee *et al.*, 1981). The composition of the peptides was confirmed by amino acid analysis on a Durrum D-500 column (molar proportions: for peptide I, Ala:Asp:Lys 1.03:4.02:0.95; for peptide II, Ala:Asp:Lys 0.95:4.10:0.95; for peptide III, Ala:Asp:Lys 0.96:4.07:0.97). [L-Asp]₂-L-Lys-Nap and [L-Asp]₃-L-Lys-Nap were judged to be free of detectable impurities by reversed-phase h.p.l.c. in 155 mM-KCl, pH 2.2, containing acetonitrile.

Dansylation. Dansylation was carried out as described by Gray (1972).

Fig. 3. Reversed-phase h.p.l.c. analysis of Gly-[L-Asp]₄-L-Lys-Nap breakdown products

(a) A 125 nmol portion of peptide was incubated at 37 °C with a portion of the enteropeptidase pool from Fig. 1 in a total volume of 200 μ l. After 1 h the reaction was stopped (see the Experimental section) and a 25 μ l portion was injected on to the column. Absorbance was read at 254 nm. —, Conc. of solvent B in A+B. Peaks 1 and 2, breakdown products; peak 3, undigested substrate. (b) Fragmentation pattern of Gly-[L-Asp]₄-L-Lys-Nap obtained by incubating the peptidase pool from Fig. 2 with the peptide substrate. Peak 1, [L-Asp]₂-L-Lys-Nap; peak 2, [L-Asp]₃-L-Lys-Nap; peak 3, undigested hexapeptide. (c) Reversed-phase h.p.l.c. of a mixture of synthetic [L-Asp]₂-L-Lys-Nap (corresponding to peak 1), [L-Asp]₃-L-Lys-Nap (peak 2) and Gly-[L-Asp]₄-L-Lys-Nap (peak 3). About 1 μ g of each peptide was injected.

RESULTS

A sample of the enteropeptidase pool (Fig. 1) was incubated with a solution of the peptide substrate containing approx. 125 nmol of Gly-[L-Asp]₄-L-Lys-Nap, and the breakdown products were separated by reversed-

Fig. 4. Separation of dansylated hydrolysis products of Gly-[L-Asp]₄-L-Lys-Nap

The peptide was digested for 1 h as described in Fig. 3 and dansylated as described by Gray (1972). Indicated are the elution positions for Dns-Gly-Asp, Dns-Gly and Dns-Asp. The other peaks in the chromatogram were not identified, but probably correspond to mono- and di-dansylated peptide fragments. The same solvent system as in Fig. 3 was used.

phase h.p.l.c. (Fig. 3a). Two breakdown products (peaks 1 and 2, eluted at 35.6 and 36.8 min respectively) were separated from undigested substrate (peak 3, retention time 38.6 min). Amino acid analysis of the peaks (each pooled separately) identified only aspartic acid and lysine in peaks 1 and 2 (glycine was absent), but conclusive molar ratios were not obtained.

The possibility of a stepwise degradation of the peptide substrate by a combined action of aminopeptidases, which would result in the initial liberation of glycine, followed later by aspartic acid and lysine, was investigated by dansylation of the digested substrate after incubation with the enteropeptidase pool of Fig. 1.

Fig. 5. Time-course experiment showing the release of the Gly-Asp dipeptide

The dansylated fragments were analysed by reversed-phase h.p.l.c. as indicated in Fig. 4. ■, Gly-Asp; △, Asp; ●, Gly.

Analysis of the dansylated breakdown products by reversed-phase h.p.l.c. yielded a Dns-Gly-Asp fragment (Fig. 4), indicating an endopeptidase-like breakdown of the substrate to produce a Gly-Asp fragment together with an [L-Asp]₃-L-Lys-Nap tetrapeptide, which in turn would be susceptible to further cleavage by a combination of aminopeptidases A and N to yield free 2-naphthylamine.

In order to confirm the composition of peaks 1 and 2 of Fig. 3(a), a mixture of the synthetic peptides [L-Asp]₂-L-Lys-Nap, [L-Asp]₃-L-Lys-Nap and Gly-[L-Asp]₄-L-Lys-Nap was injected on to the reversed-phase column and eluted under identical conditions. The elution pattern of the synthetic peptides was indistinguishable from that of the peptide fragments that had been generated by enzymic degradation (Fig. 3c; retention times: for [L-Asp]₂-L-Lys-Nap, 35.9 min; for [L-Asp]₃-L-Lys-Nap, 37.6 min; for Gly-[L-Asp]₄-L-Lys-Nap, 38.5 min).

The endopeptidase-like degradation was further confirmed with a time-course experiment. Substrate was incubated with the enteropeptidase pool (Fig. 1), and samples were removed at intervals, freeze-dried, dansylated and analysed by reversed-phase h.p.l.c. As shown in Fig. 5, the dipeptide is liberated the most rapidly. However, the appearance of Gly-Asp does not rule out a concomitant sequential hydrolysis by aminopeptidases, since the observed glycine peak could also be directly hydrolysed from the N-terminus or from the resulting Gly-Asp fragment.

That aminopeptidases are primarily responsible for non-specific cleavage of the artificial substrate was shown by an experiment in which aminopeptidases and enteropeptidase were separated on a *p*-aminobenzamidine-substituted Sepharose (Fig. 2). The aminopeptidases do not bind to the column and can be recovered in the void volume, whereas enteropeptidase binds strongly to the column and can only be eluted by a change in pH or ionic strength. For unambiguous localization of enteropeptidase in the effluent, all fractions were tested with trypsinogen and with the synthetic substrate. The elution profile shows two

Fig. 6. Inhibition of bovine trypsinogen activation in the presence of modified activation peptides

The zymogen was activated with the enteropeptidase pool from Fig. 2 (specific activity 109 nmol of trypsin/min per mg of protein). (a) Activation in the presence of D-Ala-[L-Asp]₄-L-Lys-OMe: ●—●, 0 mM; △—△, 0.05 mM; □—□, 0.1 mM; ■—■, 0.15 mM. (b) Activation in the presence of L-Ala-D-Asp-[L-Asp]₃-L-Lys-OMe: ●—●, 0 mM; △—△, 0.135 mM; □—□, 0.27 mM. (c) Activation in the presence of L-Ala-[L-Asp]₄-L-Lys-OMe: ●—●, 0 mM; △—△, 0.11 mM; □—□, 0.22 mM. Activation was carried out at the following trypsinogen concentrations: (a) 17.7 μ M, 27.8 μ M, 38.9 μ M and 50 μ M; (b) 16.7 μ M, 27.8 μ M and 38.9 μ M; (c) 16.7 μ M, 27.8 μ M and 38.9 μ M.

distinct hydrolysis maxima for the peptide substrate (Fig. 2); the second peak coincides with the elution position of enteropeptidase, whereas the first one coincides with that of the aminopeptidases. When the peptide substrate was incubated with the pooled aminopeptidases from the affinity column, and the cleavage products were separated by reversed-phase h.p.l.c. (Fig. 3b), elution profiles identical with those shown in Fig. 3(a) were obtained, indicating that with a crude mucosal extract the substrate is degraded much faster by aminopeptidases than by enteropeptidase. Quantification of the peak areas indicated that, in the presence of a mixture of aminopeptidases, 65% of the initial substrate amount was hydrolysed to the [L-Asp]₂-L-Lys-Nap fragment, whereas the Gly-[L-Asp]₄-L-Lys fragment, generated by the action of enteropeptidase on the substrate, detected at 206 nm and eluted in the void volume of the column, represents only a few per cent of the sum of the peak areas.

Activation of trypsinogens in the presence of synthetic analogues

In order to prevent the digestion of the substrate by aminopeptidases, peptide analogues containing D-amino acid residues were investigated as alternative substrates. Since the specificity of the endopeptidase (dipeptidase), and hence the position at which the peptide has to be modified, was not known, two analogues were synthesized with either D-aspartic acid in the penultimate position or D-alanine at the N-terminus. As the effect of stereoisomeric amino acids on the affinity of enteropeptidase was unknown, a third peptide was synthesized with all amino acids in the L configuration. The specificity of these substrates was investigated by their ability to inhibit activation of bovine trypsinogen with rat enteropeptidase that had been purified by affinity chromatography.

All peptide analogues were competitive inhibitors of trypsinogen activation (Fig. 6), with inhibition constants in the micromolar range (Table 1). The K_m value of bovine trypsinogen was found to be 72 μ M. Although

enteropeptidase has been reported to possess esterolytic activity (Maroux *et al.*, 1971), no direct K_m determinations could be obtained by measuring esterase activity with a micro-pH-stat. Similar findings have been reported previously by Grant & Hermon-Taylor (1979).

DISCUSSION

Enteropeptidase, formerly known as enterokinase, plays a key role in initiating proteolytic digestion by converting trypsinogen into trypsin, which in turn activates all other pancreatic zymogens. Enteropeptidase is synthesized by the enterocytes of the proximal small intestine and incorporated into the brush-border membrane. However, unlike most other brush-border membrane hydrolases, the enzyme is much more easily solubilized from the membrane into the intestinal lumen by pancreatic proteinases (Nordström, 1972). In coeliac disease, which results in severe villus atrophy, there is a marked decrease in brush-border enzymes, whereas the amount of enteropeptidase remains unaffected (Woodley & Keane, 1972), suggesting that the association of enteropeptidase with the membrane differs

Table 1. Inhibition constants of modified activation peptides

Inhibition was measured at a substrate concentration of 38.9 μ M. The concentration of inhibitor is indicated in parentheses. K_i values are given as means $\pm$ S.E.M. ($n = 4$).

Peptide	K_i (μ M)	Inhibition (%)
L-Ala-D-Asp-[L-Asp] ₃ -L-Lys-OMe	52.0 $\pm$ 8.8	89 (0.27 mM)
D-Ala-[L-Asp] ₄ -L-Lys-OMe	50.4 $\pm$ 7.8	68 (0.15 mM)
L-Ala-L-Asp-[L-Asp] ₄ -L-Lys-OMe	75.8 $\pm$ 10.2	71 (0.22 mM)

Fig. 7. Fragmentation pattern of the synthetic enteropeptidase substrate by mucosal aminopeptidase

Abbreviations: DAP, dipeptidylaminopeptidase; APA, aminopeptidase A; APN, aminopeptidase N.

fundamentally from that of the other brush-border-membrane enzymes.

The role of pancreatic enzymes in maintaining normal levels of enteropeptidase activity is demonstrated by the fact that enzyme activity is deficient in patients with exocrine pancreatic insufficiency, but normal activities return after oral treatment with pancreatic extracts (Lentze *et al.*, 1982). However, to gain further insights into the biosynthesis and subcellular processing of human enteropeptidase, a more sensitive assay system than the present trypsinogen activation test is needed.

The *N*-terminal activation peptide liberated from all mammalian trypsinogens during activation has the amino acid sequence Xaa-[Asp]₄-Lys, which appears to be essential for the interaction with enteropeptidase and provides a specific signal for fast activation in the small-intestinal lumen (Maroux *et al.*, 1971). On this basis, synthetic peptide substrates have been developed to overcome the problems of instability and auto-activation inherent in the physiological substrate, trypsinogen.

Our experiments demonstrate two major points concerning the use of the synthetic substrate Gly-[L-Asp]₄-L-Lys-Nap: a combination of aminopeptidases is able to liberate 2-naphthylamine and thus mimics the presence of enteropeptidase, and the degradation of the substrate does not proceed sequentially from the *N*-terminus. An endopeptidase cleaves a dipeptide from the *N*-terminus, thus generating an ideal substrate for aminopeptidase A, which then degrades the remaining part of the peptide down to the Lys-Nap fragment. This in turn is split by aminopeptidase N to release the fluorophore (Fig. 7).

The occurrence of an endopeptidase (or rather a dipeptidase) that accepts such acidic peptides was an unexpected finding. The only well-characterized dipeptidase of the small-intestinal mucosa is dipeptidyl aminopeptidase IV (Kenny *et al.*, 1976), which cleaves peptides after Gly-Pro residues ('post proline cleaving enzyme'; Hopsu-Havu & Glenner, 1966) and therefore is an unlikely candidate for the hydrolysis of the artificial enteropeptidase substrate. Furthermore, in order to compare the established brush-border-membrane peptide hydrolases with the Gly-Asp-cleaving dipeptidase, this enzyme's subcellular distribution and inhibition spectrum have to be studied in more detail.

The separation of polyaspartic acid fragments, which enabled us to determine the fragmentation pattern of the synthetic substrate by aminopeptidases, was achieved by the use of reversed-phase h.p.l.c. The peptide was strongly adsorbed on the stationary phase by the 2-naphthylamine moiety and could only be eluted with an ion-pair solvent. However, a weak interaction between the aspartic acid residues and the C₁₈ support

was also observed, since increasing the number of aspartic acid residues within the peptide sequence prolonged the retention time on the column. Therefore the solvent system comprising 155 mM-KCl, pH 2.2, containing acetonitrile was an ideal system for monitoring endopeptidase-like degradation that left the 2-naphthylamine part intact and produced fragments hydrophobic enough to be retained on the stationary phase. Unfortunately, owing to the carcinogenic properties of 2-naphthylamine, it was not possible to synthesize stereoisomeric substrate analogues with 2-naphthylamine at the *C*-terminus and so we were unable to compare directly by reversed-phase h.p.l.c. the splitting of the stereoisomeric substrates and the Gly-[L-Asp]₄-L-Lys-Nap, which would have indicated whether the hydrolysis by the endopeptidase could be prevented.

The fact that the insertion of stereoisomeric aspartic acid residues into the sequence of the activation peptide did not alter its properties as a competitive inhibitor of the enteropeptidase-catalysed activation of trypsinogen suggest that these alternative substrates could probably be used for measuring enteropeptidase. However, their use has to be further investigated by resynthesizing them with an appropriate fluorophore at the *C*-terminus (e.g. 7-amino-4-methylcoumarin), and determining their specificity and hydrolysis-resistance against the aminopeptidases described in this paper.

This work was supported by Grant no. 3.841-0.79 from the Swiss National Science Foundation and the SANDOZ-Stiftung zur Förderung der medizinisch-biologischen Wissenschaften. We thank Dr. Lavanchy, Landwirtschaftliche Forschungsanstalten, Liebefeld, Switzerland, for the amino acid analysis, Mrs. C. Neufeldt for excellent technical assistance and Miss S. Lerch for typing the manuscript.

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Received 1 April 1986/25 July 1986; accepted 7 October 1986