The Catalytic Activity of Pig Pepsin C towards Small Synthetic Substrates

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A series of small peptides has been synthesized and used to investigate the activity of a minor pig pepsin, pepsin C (EC 3.4.23.3). The peptides had the general formula A-Leu-Val-His-B. B was either OMe, NH₂ or OH. With B = NH₂ hydrolysis ($k_{\text{cat.}}/K_m$) at 37°C and pH 2.07 increased as A was Ac-Ala, Ac-Tyr, Ac-Phe and Ac-Ala-Phe. The pH dependence of the hydrolysis of Ac-Phe-Leu-Val-His-NH₂ indicated the apparent pK_a values of two catalytically important groups on the enzyme as 1.42 and 4.88. Inhibition of the hydrolysis of the same peptide by Ac-Phe at pH 3.01 showed a form of mixed non-competitive inhibition. Hydrolysis of Ac-Tyr-Leu-Val-His-OMe and the corresponding amide showed non-classical kinetics, which are discussed in terms of a substrate-activating mechanism. The results are discussed with reference to observations made by other workers on pig pepsin A.

Pepsin C (EC 3.4.23.3, formerly called parapepsin II) is a proteinase accounting for 5–10% of the total peptic activity of pig stomachs and was first isolated from crude commercial pepsin (Ryle & Porter, 1959). It is formed by activation of the related zymogen (Ryle, 1960a) and both proteins have been further purified and characterized (Ryle & Hamilton, 1966). Pepsin C shows many physical, chemical and enzymic similarities to the major gastric proteinase, pepsin A; in particular it shares a similar peptide sequence at the active site (Kay & Ryle, 1971) but differs most markedly in its low activity against the peptide acetyl-L-phenylalanyl-L-di-iodotyrosine, which is a good substrate for pepsin A (Baker, 1951). It has hitherto been routinely assayed with acid-denatured haemoglobin as a substrate.

Valuable insights into the mechanism of action of pepsin A have been obtained from studies of its activity with a wide range of peptides and these studies have been extensively reviewed (Knowles, 1970; Fruton, 1970, 1976; Clement, 1973; Hofmann, 1974). Similar studies on the related pepsin C might further our understanding of the mechanism of action of acid proteinases, but for such work small peptide substrates containing a single sensitive bond are needed.

Attempts to prepare such substrates have been based on the observation (Ryle & Porter, 1959) that the alanyl-leucine (14–15) and tyrosyl-leucine (16–17) bonds in the sequence -Ala-Leu-Tyr-Leu-Val-Cys(O₃H)- and the phenylalanyl-phenylalanine (22–23) bond of the B chain of oxidized insulin were readily hydrolysed (Ryle *et al.*, 1968). Acetyl- and benzoyloxycarbonyl-dipeptides containing these bonds

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proved to be very poor substrates or totally resistant; they also suffered from being insoluble at acid pH values.

Three peptides which are quite good substrates for pig pepsin A were kindly provided by Professor J. S. Fruton: Z-His-X-Phe-OMe, where X = Leu, Phe or Tyr. They have been tested and were very poor substrates for pepsin C at pH 2.0 or pH 3.0.

Ryle *et al.* (1968) showed that the peptide Z-Ala-Leu-Tyr-Leu-Val-Cys(Bzl)-NH₂, although of low solubility, was hydrolysed by pepsin C. Subsequently A. P. Ryle (unpublished work) found that the shorter peptide Ac-Tyr-Leu-Val-Cys(O₃H) was a moderately good substrate at pH 2.0 ($k_{\text{cat.}}$ approx. 17 s^{-1} , K_m approx. 1.8 mM) and was suitably soluble. There were difficulties in the synthesis of a peptide containing tyrosine and cysteic acid and it seemed possible that a basic group in place of the cysteic acid residue might give a substrate with a lower K_m for this very acidic enzyme, so our attention has turned to analogous peptides containing histidine. An established route for the synthesis of such peptides is provided by the modification of Schafer & Carlsson (1972) of the picolylester method of Burton & Young (1971). In this modification a blocked C-terminal histidine residue serves in place of a picolyl group to allow the purification of the peptide on an ion-exchanger after each coupling.

Experimental

Materials

Pepsin C was prepared from pepsinogen C as described by Kay & Ryle (1971). *NN*-Dimethylformamide was from British Drug Houses Ltd., Poole, Dorset, U.K., and was purified and tested as described by Stewart & Young (1969), except that

potassium carbonate was used for the initial removal of free acid. Amberlite IR-4B, and Amberlyst 15 resins and trifluoroacetic acid were also from British Drug Houses. *NN'*-Dicyclohexylcarbodi-imide was from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. Hydroxybenzotriazole was synthesized from chloronitrobenzene and hydrazine hydrate (G. T. Young, personal communication); *O*-benzyl-tyrosine was prepared from the L-amino acid as described by Schnabel (1967).

Methods

Peptide synthesis. The peptides were synthesized by stepwise addition of residues as their *t*-butyloxycarbonyl derivatives starting from the C-terminal histidine methyl ester. Coupling was achieved in dimethylformamide by the two-stage procedure with *NN'*-dicyclohexylcarbodi-imide and hydroxybenzotriazole (König & Geiger, 1970). The products were isolated by chromatography on Amberlyst 15 resin (Schafer & Carlsson, 1972). The *t*-butyloxycarbonyl protecting groups were removed by treatment with trifluoroacetic acid. Boc-Leu-Val-His-OMe was synthesized and provided the starting material for all the further syntheses, as well as a small sample of authentic Leu-Val-His-OMe and, by saponification, the tripeptide. After addition of the last (*N*-terminal) residue and deprotection, trifluoroacetate was exchanged for acetate by passage through Amberlite IR-4B in acetic acid before acetylation of the amino group with acetic anhydride. The benzyl group protecting the tyrosine side chain was removed by catalytic hydrogenation in 80% acetic acid. The peptide amides were prepared from their methyl esters by ammonolysis with dry ammonia in methanol. The purity of each intermediate *N*-protected peptide ester was checked by thin-layer chromatography but these intermediates were not crystallized.

In an early preparation of Ac-Tyr-Leu-Val-His-OMe considerable saponification occurred during washing with NaHCO_3 solution. Advantage was taken of this to isolate the corresponding peptide with free carboxyl group by preparative thin-layer chromatography in system G3 containing 0.25 vol. of methanol.

Acetyl-L-phenylalanine was prepared by the reaction of L-phenylalanine with acetic anhydride, the pH being maintained at 8.0 with 2.0M-NaOH in a pH-stat. The solution was then acidified with 5.7M-HCl, and cooled to precipitate the product. Yield 90%; m.p. 168–169°C (lit. 169–170°C), $[\alpha]_D^{25} 45.5^\circ$ (c 2% in EtOH) (Found: C, 63.9; H, 6.53; N, 6.65. Calc.: C, 63.8; H, 6.28; N, 6.76%).

Thin-layer chromatography. Silica gel (Kieselgel HF, Typ 60 from Merck, Darmstadt, Germany) and the solvent systems E3 [methanol/chloroform (1:3, v/v)], E4 [methanol/chloroform (1:9, v/v)] and G3

[pyridine/acetic acid/water/ethyl acetate (20:6:11:11, by vol.)] were used. Peptides were located by ninhydrin, Pauly and chlorine stains (Dawson *et al.*, 1969). Boc-peptides gave no reaction with ninhydrin at 70°C, 10min, but were readily detected on heating at 100°C.

Electrophoresis on paper. Buffer systems at pH 2.0 [1.5M-formic acid/2.0M-acetic acid (1:1, v/v)] and pH 3.6 (10ml of pyridine and 100ml of acetic acid in 2 litres of solution) were used, and the papers were cooled under white spirit. Peptides were detected (Offord, 1969) by ninhydrin and Pauly reactions and by hypochlorite/starch/KI (aqueous NaOCl was used: 1% available chlorine). Boc-peptides could not be detected on paper with ninhydrin.

Assays of enzymic activity. Routine assays with haemoglobin or Ac-Phe-Tyr(I_2) as substrate were done as described by Ryle (1970). Tests for activity of pepsin C with new peptides were sometimes performed by substituting the peptide for Ac-Phe-Tyr(I_2) at a suitable concentration but were more usually done by the automated method described below.

Automatic recording of peptide hydrolysis. The activity of pepsin C towards the new peptide substrates was measured by using the proportioning pump and photometer of a Technicon Auto-Analyzer to monitor the hydrolytic reaction continuously, as in the methods described by Lenard *et al.* (1965) and by Cornish-Bowden & Knowles (1965). Standard tubing of internal diameter 0.03in (0.76mm) and 0.035in (0.89mm) was used for the nitrogen and sample lines respectively, and Solvaflex tubing was used for the ninhydrin reagent (0.035in, 0.89mm) and return line (0.025in, 0.64mm). The ninhydrin reagent was prepared as for the reagent of Moore & Stein (1954) except that the final solution contained 550ml of 2-methoxyethanol, 85ml of the acetate buffer, 4.8g of ninhydrin and 0.48g of hydrindantin per litre. The concentration of 2-methoxyethanol had to be thus decreased because of instability of the Solvaflex tubing. The reagent was stored in an amber bottle under N_2 .

Experimental determinations were performed by using 0.05M-citrate buffers, from pH 2.0 to 5.5. At pH values below 2, HCl was added to give the required pH, the concentration of total citrate being maintained. Where possible the ionic strength of the buffers was adjusted to 0.175M with sodium chloride. The concentration of pepsin C solutions was determined from their absorbance at 280nm; $A_{280}^{1\text{mg/ml}} = 1.3$ for a freeze-dried sample, mol.wt. 36000 (Ryle & Hamilton, 1966).

Results

Peptides

The peptides studied are shown in Table 1. Amino acid analyses, thin-layer chromatography and paper

Table 1. *Properties of peptides*

Potential substrates	Melting point (°C)	R_F		Electrophoretic mobility (R_{Lys})	
		In E4	In G3	pH 2.0	pH 3.6
Ac-Ala-Leu-Val-His-OMe	138–141	0.19	0.14	0.44	0.5
Ac-Ala-Leu-Val-His-NH ₂		0.02	0.05	0.44	0.5
Ac-Tyr-Leu-Val-His-OMe		0.19	0.26	0.43	0.49
Ac-Tyr-Leu-Val-His-OH		0.08	0.01	0.39	0.21
Ac-Tyr-Leu-Val-His-NH ₂	159–161	0.02	0.10	0.44	0.49
Ac-Phe-Leu-Val-His-OMe	187–188 (decomp. 170)	0.31	0.39	0.44	0.49
Ac-Phe-Leu-Val-His-NH ₂	223–225 (decomp. 190)		0.14	0.43	0.49
Ac-Ala-Phe-Leu-Val-His-NH ₂	(decomp. 225)		0.09		0.4
Peptide products					
Leu-Val-His-NH ₂			0.02	0.93	1.05
Leu-Val-His				0.83	0.70
Ac-Tyr				–0.01	–0.3

electrophoresis of the final products indicated their homogeneity and the expected composition and charge of the molecule. Elemental analyses were not very satisfactory in some cases, probably because of contamination with a small amount of material from the Amberlyst resin. The resin, despite several regeneration cycles, still yielded a little contaminant during elution of the product.

Purity of the pepsin C preparation

The enzyme was examined by electrophoresis in polyacrylamide gel (7% total acrylamide, 0.02M-acetate buffer, pH 5.6, 11 V/cm, 60 min). Duplicate gels were stained with Coomassie Blue for detection of proteins and were stained for proteinase activity with 2% haemoglobin at pH 1.7 as substrate, essentially by the method of Uriel (1960).

The protein appeared as a double band at 3.5 and 3.2 cm, accounting for 60 and 30% of the area of a densitometric scan of the gel. The remaining 10% was accounted for by a band at 4.4 cm, the position occupied by pepsin A in a gel run in parallel. Proteinase activity was detectable at the sites of pepsin A and the double band, but the resolution of the stain for proteinase is not good enough to show conclusively that both the components of the double band were proteolytically active.

Double bands, rather more widely separated than those found here, have also been observed for pepsinogens A and B and for pepsin B (Ryle, 1965); both components of the pepsin B band were enzymically active.

The extent of contamination of pepsin C with pepsin A was more accurately measured by determining the relative activities of the preparation with haemoglobin and with the peptide Ac-Phe-Tyr(I₂). No preparation of the enzyme (or its zymogen) has been obtained devoid of (potential) activity with this

peptide, but, if one makes the extreme assumption that pure pepsin C has no such activity one can calculate, from the relative activities of a preparation and from the known relative activities of pepsin A, a value for the maximum possible extent of contamination. The value thus obtained for the preparation used in this work was 10%; other preparations have given a value of 3–4%, so that the present preparation must contain 7–10% of pepsin A.

Products of hydrolysis of the peptides with pepsin C

Samples, equivalent to 25 nmol of original substrate, from incubation mixtures (0.5 mM-substrate, 0.1 mg of enzyme/ml, pH 2.07, 37°C, 15–20 h) were subjected to electrophoresis on paper at pH 2 and 3.6 at about 30 V/cm for 60–75 min. The tyrosine-containing peptide amide and ester each showed only two product spots, corresponding to Leu-Val-His-amide or ester and Ac-Tyr by comparison with authentic samples, when stained with ninhydrin and Pauly reagents (the electrophoretic mobilities are shown in Table 1). No residual substrate was detected. A single product spot with the mobility of Leu-Val-His-amide or ester was observed for the other peptides, suggesting a single cleavage in these peptides also. This was confirmed for Ac-Phe-Leu-Val-His-amide by eluting products of an incubation mixture which had been subjected to electrophoresis at pH 2. A marker strip stained with ninhydrin showed the presence of Leu-Val-His-amide, which, on elution and hydrolysis, yielded the expected three amino acids in equimolar proportions. High loading of an authentic sample of Ac-Phe, which was detected by hypochlorite/starch/KI staining, indicated the locality of the other presumed product, and hydrolysis of the eluate, followed by amino acid analysis, showed only phenylalanine in this fraction. No amino acids were significantly present in any other fraction of the

electrophoretogram, including the region which would have contained undigested material.

All of the peptides were hydrolysed by pepsin C, but Ac-Ala-Leu-Val-His-OMe and the corresponding amide required very high concentrations of enzyme (0.5 mg/ml) for hydrolysis to be detectable after 16 h incubation, and undigested substrate still remained. Hydrolysis of these peptides was therefore not investigated further.

Stability of the peptides

During these studies considerable conversion of the tyrosine peptide ester into the corresponding free acid has been observed, in neutral or mildly acid solutions, as well as in the presence of NaHCO_3 . No attempt has been made to measure the rate of conversion but it is our impression that this ester is unusually labile; the lability may be due to the nearby imidazole and phenolic groups. Ester solutions for kinetic work were therefore freshly prepared. The amide analogues were synthesized and proved satisfactorily stable.

Kinetics of the hydrolysis of peptides by pepsin C

Initial velocities were measured from the initial gradients of the Auto-Analyzer traces. Since we do not have enough data to know whether the errors are normally distributed or how the data should be correctly weighted for a least-squares fitting procedure, and since the values of substrate concentration must

themselves be subject to error, there is no sound basis for estimating the kinetic parameters by a parametric method. We have therefore made the estimates by the method of Eisenthal & Cornish-Bowden (1974) as later modified (Cornish-Bowden & Eisenthal, 1978) using a computer program written by Dr. G. L. Atkins. The program also supplied 95% confidence limits.

The parameters so obtained are shown in Table 2. Because of the limited quantities or the low solubility of the peptides most of the determinations were made at substrate concentrations below K_m , so that the confidence limits are rather wide. In such circumstances an estimate of $k_{\text{cat.}}/K_m$ taken from the initial slope of a plot of initial velocity versus substrate concentration should be better than the estimate of this parameter obtained as the ratio of the separate estimates of $k_{\text{cat.}}$ and K_m . Values of $k_{\text{cat.}}/K_m$ were therefore found from the slope obtained by fitting a second-degree polynomial to the $[S]$ and v data. These values are given in Table 2.

The tyrosine-containing peptide amide and ester both gave data that did not fit the Michaelis-Menten equation well. This is most clearly seen in the Hanes plot for the ester (Fig. 2). The Figure includes data obtained on five separate occasions, scaled to give precisely the same velocity at 0.1 mM-substrate, but scaling in other ways gave essentially the same kind of plots. Similar deviation from Michaelis-Menten kinetics was also observed for the analogous amide at pH 2.1 in a single data set of 11 substrate concentra-

Table 2. *Kinetic parameters for the hydrolysis of peptides by pepsin C at pH 2.1 and 37°C*
a, Ratio of individual $k_{\text{cat.}}$ and K_m . b, Values from initial slope of plot of v versus $[S]$.

Substrate*	Range of $[S]$ (mM)	K_m (mM)†	$k_{\text{cat.}}$ (s^{-1})†	$k_{\text{cat.}}/K_m$ ($\text{s}^{-1} \cdot \text{mM}^{-1}$)	
				a	b
Ac-Tyr-Leu-Val-His-OH	0.2–1.0	0.82 (0.56–1.06)	1.08 (0.92–1.27)	1.31	1.07
Ac-Tyr-Leu-Val-His-NH ₂	0.1–2.0	4.31 (3.53–5.70)	4.45 (3.51–5.50)	1.03	0.87
	0.1–0.5	0.82 (0.49–1.22)	1.07 (0.69–1.37)	1.31	1.16
Ac-Tyr-Leu-Val-His-OMe	0.025–1.00	1.71 (1.46–2.14)	5.76 (5.34–6.76)	3.36	2.77
	0.025–0.15	0.28 (0.24–0.31)	1.06 (0.99–1.22)	3.81	3.45
Ac-Phe-Leu-Val-His-NH ₂	0.5–1.4	1.84 (1.07–2.52)	6.12 (4.34–7.79)	3.32	2.86
Ac-Phe-Leu-Val-His-OMe	0.10–1.6	1.84 (1.67–2.17)	5.13 (4.79–5.79)	2.78	2.15
Ac-Ala-Phe-Leu-Val-His-NH ₂	0.075–0.375	0.70 (0.48–1.39)	7.01 (5.48–11.86)	10.1	9.11
Ac-Ala-Leu-Val-His-NH ₂ Ac-Ala-Leu-Val-His-OH }	Very slow hydrolysis				

* Hydrolysis occurred exclusively at the amino side of the leucine residues.

† 95% confidence limits in parentheses.

tions. The peptide ester showed the same kind of deviation at pH 2.6 and 2.9 as well. Table 2 shows parameters for these two peptides taken from the whole range of substrate concentrations and from the lower part of the range only. The other peptides, including Ac-Tyr-Leu-Val-His-OH, did not show any obvious deviation from classical kinetics in the range of concentrations studied.

Dependence of catalysis on pH

The peptide Ac-Phe-Leu-Val-His-NH₂ was used for these studies as it had a fairly favourable value of $k_{\text{cat.}}/K_m$ at pH 2.1 and apparently simple kinetics, at least in the range of substrate concentrations investigated. The buffers used are described under 'Methods'.

Fig. 2 shows the dependence of $V_{\text{max.}}$ and $V_{\text{max.}}/K_m$ upon pH. A theoretical bell-shaped curve for the equation $y = x \cdot K_1 H / (H^2 + K_1 H + K_1 K_2)$, where y is the observed value of $k_{\text{cat.}}$ or $k_{\text{cat.}}/K_m$, K_1 and K_2 are two acid dissociation constants and x is the maximum value y would reach if $K_1 \gg K_2$, was fitted to the data by reiterative adjustment of assumed initial values for K_1 , K_2 and x with a computer program written by Dr. G. L. Atkins.

Fig. 1. Hydrolysis of Ac-Tyr-Leu-Val-His-OMe by pepsin C. Hydrolysis took place at 37°C and pH 2.1 at an enzyme concentration of 1.4 µg/ml. Data from five separate experiments were normalized to the same velocity at 0.1 mM-substrate. Error bars show the s.d. of the five observations for which replicates ($n = 2-5$) were thus obtained. At 0.2 mM-substrate (upper plot) and at 1.0 mM-substrate the error was too small to draw; at some other points the symbol at the mean has been omitted for clarity. In both plots the line is that generated by the Michaelis equation with the parameters obtained from all the data (Table 1).

Fig. 2. pH-dependence of (a) $V_{\text{max.}}$ and (b) $V_{\text{max.}}/K_m$ for the hydrolysis of Ac-Phe-Leu-Val-His-NH₂ by pepsin C. Hydrolyses were performed in 0.2M-citrate buffers at 37°C, with a final enzyme concentration of 1.4 µg/ml. The continuous lines represent theoretical bell-shaped curves fitted to the data.

The values of pK of catalytically important groups corresponding to the values of K_1 and K_2 obtained from the fitting procedure were found to be 1.42 (± 0.06) and 4.88 ($+0.02$, -0.03) for the free enzyme and 1.37 ($+0.09$, -0.0) and 4.87 ($+0.03$, -0.04) for the enzyme-substrate complex. Thus the apparent pK values of the groups on the enzyme do not change significantly on binding the substrate molecule.

Inhibition of the hydrolysis of Ac-Phe-Leu-Val-His-amide by Ac-Phe

The experiments were carried out at pH 3.01, where the uninhibited rate was greatest.

A preliminary study with 1.0 mM-substrate and various concentrations of acetyl-L-phenylalanine showed that it would be useful to study the inhibition over a range of inhibitor concentrations up to 30 mM.

Experiments were performed with the concentration of Ac-Phe varying from 0 to 30 mM and substrate from 0.5 to 1.5 mM. Parameters were determined by

the direct linear plot, but a double-reciprocal plot was constructed on the basis of these results to determine the inhibition type. The inhibition is mixed, according to the nomenclature of Cornish-Bowden (1976a), both K_m and V_{max} being affected by the presence of the inhibitor. The values of K_i and K_i' [K_i (slope) and K_i (intercept) of Cleland (1963)] were 21.7 (+3.6, -3.0) mM and 46.4 (+7.3, -5.9) mM respectively.

Discussion

Tyrosine-containing peptides

Comparison of the kinetic data for the substrates of pepsin C is complicated by the deviation from Michaelis-Menten kinetics detected for Ac-Tyr-Leu-Val-His-NH₂ and for the corresponding methyl ester, and it is necessary first to consider whether this deviation is a real effect. Replicate determinations are not useful when data are analysed by the method of Eisenthal & Cornish-Bowden (1974), so they were not, in general, made. Those shown in Fig. 1 are provided by pooling five sets of data obtained on different occasions. Although the deviation from the theoretical curve in the plot of v against $[S]$ seems slight it would be difficult to argue that the points in the Hanes plot (Fig. 2b) can be fitted by a single straight line. We conclude that the Michaelis-Menten model does not describe these data. Further support for this belief comes from examination of the signs of the residuals of all the data when compared with the velocities given by the estimates of K_m and V_{max} obtained by the direct linear plot. The 27 signs are found in five runs (7+, 18-, +, -, 2+). The probability of finding such a distribution if the errors were random and the data were being fitted to the correct model is about 2×10^{-4} . Indirect support for the reality of the deviation comes from our observation of the same phenomenon, albeit with fewer data, for the same substrate at two different pH values and for the analogous amide at pH 2.1.

Accepting that the data do not fit the Michaelis-Menten equation we must now examine whether the deviation may be some kind of artifact. Care was taken to see that the sensitivity of the photometer and chart-speed of the recorder were not changed during a series of rate measurements, so that no consistent error can have been introduced by such changes. It is difficult to see how we could have consistently underestimated velocities in the middle range relative to the higher and lower ones. The fact that we did not find abnormal curves for the other substrates also argues against there being some kind of consistent error in the observations.

A deviation of the kind observed requires a minimum of three enzyme-substrate interactions (Teipel & Koshland, 1969; Bardsley & Childs, 1975), the simplest scheme being a summation of a classical

hyperbolic curve and a sigmoid curve, each produced by a separate enzyme-substrate reaction, such as might occur if the substrate or the enzyme was not homogeneous. The two tyrosine-containing peptides appeared chemically homogeneous as judged by their behaviour on thin-layer chromatography in two solvent systems. The method of synthesis used (activation of Boc-amino acids with dicyclohexylcarbodiimide in the presence of hydroxybenzotriazole) is believed to give product with very little racemization, and the fact that the tyrosine-containing substrates could be completely hydrolysed to acetyl tyrosine and the tripeptide amide or ester further suggests that neither the tyrosine nor the leucine had become racemized. We do not think therefore that heterogeneity of the substrates is a likely explanation for the deviation observed.

The enzyme preparation, however, is known not to be homogeneous. On electrophoresis in polyacrylamide gel two major components were detected which we believe to be two forms of pepsin C, analogous to the two forms which have been observed for pepsin B, its zymogen and for pepsinogen A. The contamination by not more than 10% of pepsin A cannot account for the abnormal behaviour observed because pepsin A, tested at 20 µg/ml with 0.5 mM-tyrosine-containing substrates, gave very slow hydrolysis, which could just be detected on incubation for 18 h; the pepsin A contamination could not have contributed more than 1% of the rate of hydrolysis of these peptides observed.

We therefore suggest that the abnormal kinetics are a true property of the pair of proteins constituting 90% of the preparation.

Comparison of peptides

The effect of changing the amino acids at the amino side of the sensitive bond is most easily seen by comparing the values of the specificity constant, k_{cat}/K_m for the amides. This parameter is not greatly different for Ac-Tyr-Leu-Val-His-NH₂ whether calculated from the whole range of substrate concentrations or from the lower ones only, so that the deviation from classical behaviour will not interfere in the comparison. The value of the specificity constant increases as Ala is replaced successively by Tyr, Phe and Ala-Phe.

This, taken with the resistance of dipeptide substrates to hydrolysis by pepsin C (Ryle *et al.*, 1968), suggests that pepsin C, like pepsin A (Fruton, 1970, 1976) has an extended binding site. There must, however, be significant differences in the structure of this site for the two enzymes as shown by their different substrate specificities. Pepsin C hydrolyses small peptides in which tyrosine is the acyl moiety of the sensitive bond, whereas such peptides are poor substrates for pepsin A (Fruton, 1970; Ryle & Auffret, 1979). Likewise in a quantitative study Pedersen (1977) has shown that the major sites of attack of

pig pepsin A on the B-chain of oxidized insulin are at Leu-Val, Leu-Tyr and Phe-Tyr bonds and the Tyr-Leu bond is relatively resistant.

The resistance of Ac-Ala-Leu-Val-His-OMe or the corresponding amide to hydrolysis by pepsin C is surprising in view of the relatively rapid hydrolysis of the Ala-Leu (14-15) bond in the B-chain of oxidized insulin (Ryle & Porter, 1959). It may be significant that although in that work the Tyr-Leu (16-17) bond was also found to be readily hydrolysed by pepsin C, no leucyltyrosine, which would be expected as a product if both cleavages occurred in the same molecule, was detected. It is possible that cleavage at either site inhibits cleavage at the other, may be because of the requirement for an extended binding site.

The studies of the pH-dependence of the catalytic activity of pepsin C (Fig. 2) were made with the peptide Ac-Phe-Leu-Val-His-amide, which displays simple saturation kinetics in the concentration range used. They show that K_m remains almost constant whereas k_{cat} varies, so that for pepsin C, as with pepsin A, all the enzyme forms are in equilibrium, the rate-determining step being the regeneration of free enzyme (Cornish-Bowden, 1976b). The apparent pK_a values of 1.42 and 4.88 found are close to those of about 1 and 4.5, which have been found for pepsin A (Denburg *et al.*, 1968; Cornish-Bowden & Knowles 1969; Clement *et al.*, 1968). One of the ionizable residues in the active site of pepsin C has been shown to be an aspartic acid residue (Kay & Ryle, 1971), and both are known to be aspartic acid residues in pepsin A (Fry *et al.*, 1968; Bayliss *et al.*, 1969; Hartsuck & Tang, 1972).

The studies of inhibition of hydrolysis of Ac-Phe-Leu-Val-His-NH₂ performed at pH 3, showing mixed inhibition by the acyl product, are consistent with an ordered release of products, the acyl product leaving first, and implying the existence of some kind of amino-enzyme intermediate. Inhibition by the amino product would then be expected to be competitive, but this could not be tested with the ninhydrin method used for following the progress of the reactions. The existence of an amino-enzyme intermediate is consistent with transpeptidation of the amino-transfer type, as already observed for pepsin C (Ryle, 1960b), and with a change in k_{cat} , but not in K_m when the amino portion of the substrate is changed from the amide to the ester (Table 2).

Comparison of the data in Table 2 for the tyrosine peptides at low substrate concentrations shows, however, that change from amide to ester results in no change in k_{cat} , and a decrease in K_m . This suggests that for the tyrosine peptides at low concentrations the rate-determining step is not the same as that for the phenylalanine analogues.

The deviation of the behaviour of the tyrosine peptides from the Michaelis-Menten model suggests a mechanism involving some kind of activation by

substrate. If the deviation is not an artifact due to inhomogeneity of the enzyme or substrate or both then binding of three substrate molecules to the enzyme is required. It is not necessary that all of each tetrapeptide should be in contact with the enzyme surface, however. Activation of the peptidase activity of pig pepsin A by small substrate-like molecules has been reported by Wang & Hofmann (1976).

No inhibition or activation of the hydrolysis of Ac-Tyr-Leu-Val-His-OMe was found on adding 0.5 mM-Ac-Ala-Leu-Val-His-OMe, which is not hydrolysed under the conditions used, to incubations containing low concentrations of substrate. Therefore the alanine peptide is either not bound to the enzyme or inhibitory and activating effects are balancing each other, and this suggests that it is the aromatic part of the molecule that is responsible for the activation effect when an equivalent excess of substrate is present.

It is evident, but unsurprising, that there are strong similarities between the mechanisms of action of pepsin C and pepsin A. However, the catalytic action of pepsin A has been shown to be highly complex, and a single pathway for the hydrolysis of substrate molecules appears to be unlikely, the mechanism depending on the particular substrate being considered (Hofmann, 1974). Insufficient evidence is yet available to allow one to draw even this conclusion about the mechanism of action of pepsin C.

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