An Active Site Peptide from Pepsin C

By J. KAY* AND A. P. RYLE

Department of Biochemistry, University of Edinburgh Medical School,
Teviot Place, Edinburgh EH8 9AG U.K.

(Received 4 February 1971)

Porcine pepsin C is inactivated rapidly and irreversibly by diazoacetyl-DL-norleucine methyl ester in the presence of cupric ions at pH values above 4.5. The inactivation is specific in that complete inactivation accompanies the incorporation of 1 mol of inhibitor residue/mol of enzyme and evidence has been obtained to suggest that the reaction occurs with an active site residue. The site of reaction is the β -carboxyl group of an aspartic acid residue in the sequence Ile-Val-Asp-Thr. This sequence is identical with the active-site sequence in pepsin and the significance of this in terms of the different activities of the two enzymes is discussed.

Pepsin C (EC 3.4.4.2) (formerly called parapepsin II), one of the minor gastric proteases of the pig (Ryle & Porter, 1959), has been shown to be very similar in its physical, chemical and enzymic properties to the major porcine enzyme, pepsin (Ryle & Hamilton, 1966). The most notable difference between the two enzymes is the apparent inability of pepsin C to catalyse the hydrolysis of a small peptide substrate of pepsin, acetyl-L-phenylalanyl-L-di-iodotyrosine and several similar acetylated peptides (Ryle, Leclerc & Falla, 1968) so that it was of interest to investigate the nature of the active site of pepsin C and make a comparison with the active site of pepsin. Studies of pH-rate profiles (Zeffren & Kaiser, 1967; Denburg, Nelson & Silver, 1968; Clement & Snyder, 1966; Cornish-Bowden & Knowles, 1969) have suggested that at least one (and probably a second) carboxyl group is involved directly in the catalytic activity of pepsin. The technique of affinity labelling has been applied to pepsin by using aliphatic diazo-ketones as the type of quasi-substrate likely to react with an active-site carboxyl group (Stepanov & Vaganova, 1968; Fry, Kim, Spona & Hamilton, 1968; Bayliss, Knowles & Wybrandt, 1969). Since pepsin C is so similar to pepsin in many respects, it was reasonable to assume that a diazo-ketone might be an effective irreversible inhibitor of the minor enzyme. Diazoacetyl-DL-norleucine methyl ester was first introduced as an inhibitor of pepsin by Rajagopalan, Stein & Moore (1966a) and this compound was selected as a likely inhibitor of pepsin C.

MATERIALS

Pepsinogen C. The zymogen was isolated from porcine fundic mucosae by the method of Ryle & Hamilton (1966).

* Present address: Department of Biochemistry, Marquette School of Medicine, Milwaukee, Wis., U.S.A.

Diazoacetyl-DL-norleucine methyl ester. Benzyloxycarbonylglycylnorleucine methyl ester was prepared by coupling benzyloxycarbonylglycine and norleucine methyl ester by using *NN'*-dicyclohexylcarbodi-imide. The product was obtained as an oil, and the benzyloxycarbonyl group was removed by treatment with hydrogen bromide in acetic acid without prior crystallization. The material obtained was recrystallized from methanol-ether and had a m.p. of 119°C [Rajagopalan *et al.* (1966a) quote m.p. 119°C]. Amino acid analysis on a hydrolysed sample of the dipeptide ester showed a glycine/norleucine molar ratio of 1:1. An elemental analysis was very kindly performed by Dr J. W. Minnis of this Department (Found: C, 38.1; H, 6.7; N, 9.9; Br, 28.0; $C_9H_{11}N_2O_3Br$ requires C, 38.2; H, 6.8; N, 9.9; Br, 28.2%).

Diazotization of the dipeptide ester was performed under the conditions described by Rajagopalan *et al.* (1966a). The yellow solid obtained was filtered off, washed with ice-cold water and dried *in vacuo*. The m.p. was 50°C [Rajagopalan *et al.* (1966a) quote m.p. 50°C]. The overall yield of diazoacetyl-DL-norleucine ester based on glycine was 14%.

Diazo[^{14}C]acetyl-DL-norleucine methyl ester. [$U-^{14}C$]glycine and [$1-^{14}C$]glycine (0.5 mCi of each, obtained from The Radiochemical Centre, Amersham, Bucks., U.K.) were diluted with 1 g of unlabelled glycine and converted into glycylnorleucine methyl ester by the synthetic route described above. The yield of dipeptide ester was 760 mg (m.p. 119°C). Amino acid analysis on a hydrolysed sample showed a glycine/norleucine molar ratio of 1.06:1.00. A check on the purity of the radioactive dipeptide ester by paper electrophoresis at pH 2.0 (40 V/cm) with markers of unlabelled dipeptide ester, glycine and norleucine ester revealed no free glycine or norleucine ester. The dipeptide ester was diazotized as already described.

Haemoglobin. Bovine Haemoglobin Enzyme Substrate Powder was purchased from the Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K., and was dialysed before use to remove low-molecular-weight contaminants.

Carboxypeptidase A. Carboxypeptidase A (treated with di-isopropyl phosphorofluoridate, dialysed and recrystallized) was purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. Portions of this suspension were pre-

pared for use by treatment under the conditions described by Ambler (1967).

Pepsin. The enzyme was purified from commercial crystalline pepsin by chromatography on DEAE-cellulose (Lee & Ryle, 1967) and by exclusion chromatography on Sephadex G-100.

Buffer solutions. All buffer solutions were prepared by using the sodium salts of A.R.-grade reagents. The concentration refers to the total molarity of the buffering component.

METHODS

Activity determination with haemoglobin. Assays of activity against haemoglobin were made in duplicate at 37°C and pH 1.7 by the method described by Ryle (1960) based on that of Anson (1948). The increases in extinction of the deproteinized solutions at 280 nm were converted into proteolytic units, [P.U.]^{HB} by reference to a standard curve prepared for purified pepsin C (Ryle, 1970). The acid used for acidification of the haemoglobin for assay of the enzyme dissolved in water was 0.30 M-HCl.

Determination of total nitrogen. Nitrogen was determined by the micro-Kjeldahl technique (Chibnall, Rees & Williams, 1943) on samples containing approx. 1 mg of protein.

Electrophoresis in starch gel. This was performed in horizontal gels in 0.05 M-citrate buffer, pH 5.6, by the method of Smithies (1955). The detection of protein by the chlorine method and of activity against haemoglobin were achieved as described by Ryle (1965).

Paper electrophoresis. High-voltage paper electrophoresis was carried out on Whatman no. 1 chromatography paper in buffers of the following compositions: pH 2.0, 0.75 M-formic acid-1.0 M-acetic acid; pH 3.6, pyridine-acetic acid-water (1:10:190, by vol.); pH 6.5, pyridine-acetic acid-water (25:1:475, by vol.). Electrophoresis was performed under 'white spirit' as coolant after the method of Michl (1951). After drying the papers (60°C, 40 min), amino acids and peptides were detected with ninhydrin (0.25%, w/v, in ethanol containing 0.1% pyridine), the colour being developed at 60°C for 10 min.

Amino acid analyses. Peptides were hydrolysed *in vacuo* for 24 h or 48 h at 105°C in 5.7 M-HCl and dried *in vacuo*. A Locarte amino acid analyser was used for analyses by the accelerated method of Spackman (1963).

Detection of radioactivity. Scintillation counting was performed on either a Nuclear Enterprises' Scintillation Counter NE8304 or a Packard Tri-Carb Liquid-Scintillation Counter, model 314EX, with Nuclear Enterprises Scintillant C NE572 in dioxan or NE233 in toluene, as the scintillators.

Radiochromatogram scanning was performed on a Baird & Tatlock 'Radioactive Chromatogram Counter'.

Preparation of pepsin C. Pepsin C was prepared from pepsinogen C by incubation at pH 2.0 and chromatography of the activation mixture on SE-Sephadex under the conditions used by Rajagopalan, Moore & Stein (1966b) for isolation of pure pepsin from pepsinogen.

Reaction of pepsin C with diazoacetyl-DL-norleucine methyl ester. In typical experiments, 3.6 mg of enzyme was dissolved in 3.0 ml of HCl to give pH 2.0 or 3.0 or 0.2 M-acetate buffer of an appropriate pH and a certain volume of a 0.1 M solution of cupric acetate was added. After equilibration to 20°C, the inhibitor, diazoacetyl-DL-norleucine

methyl ester, in 0.05 ml of methanol was added (control incubations revealed no detectable loss of activity in the presence of small amounts of methanol) and samples were removed for assay of haemoglobin-digesting activity at various time-intervals. Inhibitor/enzyme molar ratios are calculated on the basis of a molecular weight of 36 000 for pepsin C.

Amino terminal analysis using fluorodinitrobenzene. This was performed under standard conditions as described by Porter (1957).

Digestion with carboxypeptidase A. The material for digestion was dissolved in 3 ml of 0.2 M-NaCl and the pH was adjusted to 8.5 on a Radiometer TTT-1 autotitrator by addition of dilute KOH. A measured volume of the prepared carboxypeptidase A solution was introduced to give, usually, a final enzyme/substrate molar ratio of 1:50 and the degradation was allowed to proceed under N₂ at 37°C and pH 8.5, the pH being maintained by addition of dilute KOH controlled by the autotitrator. Samples (0.5 ml) were removed for amino acid analysis at various time-intervals, the reaction being stopped by addition of 0.1 ml of 1 M-HCl. The amino acid analyses were performed on the supernatants obtained after sedimentation in the bench centrifuge.

Sequence studies. Determination of the *N*-terminal residue of small peptides by DNS chloride was carried out by the method of Morse & Horecker (1966). The DNS-amino acids were separated and identified either by t.l.c. on KieselGel G, with the solvent systems of Morse & Horecker (1966) or by two-dimensional chromatography on polyamide thin layers (Woods & Wang, 1967) in solvent systems 1 and 2 of Woods & Wang (1967) and a system containing ethyl acetate-methanol-acetic acid (20:1:1, by vol.) (R. Saundry, personal communication). This solvent was run in the same direction as solvent 2.

Dansyl-Edman degradations were done by the method of Gray (1967).

RESULTS

Homogeneity of pepsin C. Pepsin C prepared from pepsinogen C by the method of Rajagopalan *et al.* (1966b) was found to have a specific activity of 0.5 [P.U.]^{HB}/mg of N (cf. 0.41 for pepsin C obtained from crude commercial preparations of pepsin, Ryle & Hamilton, 1966). The value obtained was very consistent and this may indicate that this method gives better preparations of active pepsin C.

Amino acid analysis on a sample of the preparation revealed a composition very similar to that found by Ryle & Hamilton (1966) except that five, rather than four, residues of lysine were found per mol. A sample of the enzyme was submitted to electrophoresis in starch gel at pH 5.6 (1.5 V/cm, 12 h). Only a single band (which had migrated 6 cm towards the anode) was detected by the chlorine method and the single band of activity, detected by the haemoglobin method, was found at the same position.

Rajagopalan *et al.* (1966b) suggested that a useful test for homogeneity of their pepsin preparation was digestion with carboxypeptidase A because

Table 1. *Amino acids liberated (mol/mol) from pepsin C and pepsinogen C by digestion with carboxypeptidase A at pH 8.5 and 37°C*

(a) Molar ratio of pepsin C/carboxypeptidase of 50:1. (b) Molar ratio of pepsinogen C/carboxypeptidase of 50:1. (c) Molar ratio of pepsinogen C/carboxypeptidase of 350:1. No other amino acid was liberated in significant amount.

Amino acid	Time (min)	...	a				b				c				
			0	10	30	60	0	30	60	240	0	1	2	5	30
Thr			0	0.6	0.7	0.8	0	0.6	0.7	0.9	0	0	0	0.15	0.4
Gly			0	0.2	0.5	0.7	0	0.4	0.5	0.7	0	0	0	0	0.2
Ala			0	2.0	2.1	2.3	0	1.9	2.0	2.2	0	0.3	0.5	0.8	1.3
Val			0	0.2	0.5	0.7	0	0.4	0.5	0.7	0	0	0	0	0.2
Phe			0	0.8	0.9	1.0	0	0.8	0.9	1.0	0	0.1	0.2	0.3	0.6

pepsin has a C-terminal sequence of -Pro-Val-Ala. Treatment of pepsin C (and pepsinogen C) with carboxypeptidase A liberated the amino acids shown in Table 1. This suggests that the C-terminus of both enzyme and zymogen is -(Val,Gly)(Thr,Phe,-Ala,Ala). It is possible that these amino acids were released from more than one chain such as might occur if the enzyme were contaminated with peptides but since the same pattern of release of amino acids was found for both zymogen and enzyme, this would imply that the contaminant was also present in the zymogen. However, pepsinogen C has been shown to have a single N-terminal serine residue (Ryle, 1960) and the conditions of purification of enzyme and zymogen differ, so a common contaminant seems unlikely. Since the zymogen and enzyme appear to have the same C-terminal composition, activation of pepsinogen C to pepsin C (like that of pepsinogen to pepsin) apparently occurs by release of peptides from the N-terminus. Amino-terminal analysis of pepsin C by 1-fluoro-2,4-dinitrobenzene revealed 0.6 mol of DNP-serine per mol of enzyme plus 0.2 mol of DNP-isoleucine or leucine per mol (values corrected for decomposition by including internal standards). It may be that activation of pepsinogen C leaves a product heterogeneous at the N-terminus. Apart from this apparent heterogeneity, it would appear that pepsin C prepared as above is homogeneous.

Inactivation of pepsin C by diazoacetyl-DL-norleucine methyl ester. Rajagopalan *et al.* (1966a) found that the inhibition of pepsin by diazoacetyl-DL-norleucine methyl ester was greatly facilitated by the presence of cupric ions. Zollinger (1961) pointed out the ability of cupric ions to 'activate' diazo compounds and Lundblad & Stein (1969) suggested that a copper-complexed carbene intermediate is the reactive species involved.

Accordingly, our experiments were carried out in the presence of cupric ions. Control experiments showed that a cupric ions/enzyme molar ratio of 300 had no effect on the activity of the enzyme.

Fig. 1. Inactivation of pepsin C (1.2 mg/ml) by diazoacetyl-DL-norleucine methyl ester (1.5 mM) in the presence of cupric ions (3.0 mM) at ●, pH 2.0; ○, pH 3.0; ▽, pH 4.0; □, pH 5.0. Incubations at the two lower pH values were in HCl, the other two in 0.2 M-acetate buffer, all at 20°C.

The inhibition of pepsin C by a diazo compound/enzyme molar ratio of 50 in the presence of a cupric ions/enzyme molar ratio of 100 at several pH values is shown in Fig. 1. Very little inhibition was achieved at pH values below about 4.5. There was a great deal of effervescence in the samples at pH 2 and 3, attributed to decomposition of the diazo compound at low pH values. Since pepsin C is irreversibly denatured at pH values about 7, it was decided to investigate further the inhibition at pH 5.0.

The effect of varying the concentration of cupric ions with an inhibitor/enzyme molar ratio of 40 is shown in Fig. 2. It was estimated that reasonably rapid inactivation of the enzyme could be obtained

Fig. 2. Inactivation of pepsin C (1.2mg/ml) by diazoacetyl-DL-norleucine methyl ester (1.2 mM) in 0.2 M-acetate buffer, pH 5.0, at 20°C in the presence of various concentrations of cupric ions: ●, zero; ○, 0.3 mM; □, 1.2 mM; ▲, 2.4 mM.

Table 2. Inhibition of pepsin C by diazoacetyl-norleucine methyl ester at various molar ratios of inhibitor/enzyme

The enzyme concentration was 1.2mg/ml and a cupric ion/enzyme molar ratio of 30 was used. The incubation time was 1 h at 20°C in 0.2 M-acetate buffer, pH 5.0.

Inhibitor/enzyme molar ratio	Activity remaining (%)
0	100
1	90
3	71
6	59
10	44
20	20
30	8
40	0

in the presence of 30g-atoms of cupric ion/mol of enzyme and this was confirmed (Table 2).

No reactivation was observed on standing the inhibited enzyme for a further 72h nor by lowering the pH to 2.0 and standing the inhibited enzyme for 24h at this pH.

Stoichiometry of the inhibition. The experiments shown in Table 2 provided samples of pepsin C of varying degrees of inhibition. These samples were separated from small molecules (excess of inhibitor, or products of side reactions) by gel filtration on Sephadex G-25 or by dialysis. Fig. 3 shows the relation between degree of inhibition and content of norleucine determined by amino acid analysis. The slope of a linear regression of y upon x is $1.02 \pm$

Fig. 3. Relation between the degree of inhibition of pepsin C by diazoacetyl-DL-norleucine methyl ester and the incorporation of inhibitor residues into the enzyme. Incubations were as in Table 2.

0.13 and the intercept is -0.0004 ± 0.083 . A Student's test shows that the slope does not differ from the theoretical 1.0 at the 5% level of significance.

Thus, when pepsin C, at a concentration of 1.2mg/ml is incubated with diazoacetyl-DL-norleucine methyl ester, complete inactivation is produced with incorporation of one inhibitor residue per mol of enzyme. However, in some experiments with higher concentrations of enzyme (2mg/ml) and 40mol.prop. of inhibitor and 30mol.prop. of cupric ions completely inactivated enzyme was found to contain up to three inhibitor residues/molecule.

Inactivation of pepsinogen C. When the zymogen, pepsinogen C, was incubated at pH 5.0 with the inhibitor (40mol.prop.) and cupric ions (30mol.prop.), the conditions that brought about complete inactivation of the enzyme within 1h, 65% of the potential activity was destroyed and 0.65 residues of inhibitor were incorporated. This inactivation could not be attributed to prior activation of the zymogen to pepsin C with subsequent inactivation of the enzyme, as a control experiment revealed that at this pH there was very little (approx. 5%) activation of the zymogen to pepsin C. When the zymogen was incubated with diazoacetyl-DL-norleucine methyl ester at pH 7.0, there was only a 10% loss in potential activity with a concomitant incorporation of 0.1 mol of inhibitor/mol.

Reaction with denatured pepsin C. Pepsin C (1.2mg/ml), denatured by exposure to pH 10 for

30min and then incubated with diazoacetyl-DL-norleucine methyl ester under the conditions of the previous paragraph, was found to have incorporated only 0.2 residue of inhibitor/molecule.

Effect of acetyl-L-phenylalanyl-L-di-iodotyrosine on the inhibition by diazoacetyl-DL-norleucine methyl ester. Acetyl-L-phenylalanyl-L-di-iodotyrosine has been found to be a competitive inhibitor of pepsin C at pH 2.0, and has been shown to inhibit the hydrolysis of a synthetic peptide by pepsin C at pH 5.0 to an extent of about 10%. (A. P. Ryle, unpublished work). The rate of hydrolysis at pH 5.0 was too slow to permit any detailed kinetic measurements. Fig. 4 shows that acetyl-L-phenylalanyl-L-di-iodotyrosine protected pepsin C from inactivation by diazoacetyl-DL-norleucine methyl ester to an extent of 10%.

Isolation of the labelled site from inhibited pepsin C. Fig. 5 shows the inactivation of 195mg of pepsin C by diazo[^{14}C]acetyl-DL-norleucine methyl ester. The inhibited enzyme was separated from excess of

inhibitor and inhibitor degradation products (from reaction of diazoacetyl-DL-norleucine methyl ester with the solvent) by gel filtration on Sephadex G-25 in water and the protein-containing fractions were pooled and freeze-dried. Analysis for the inhibitor content revealed the presence of 1.2mol of inhibitor/mol of enzyme by both isotope and norleucine determination. Since Fig. 5 indicates that incubation was continued for 30min after almost complete inactivation had been attained, the additional 0.2 residue could be due to non-specific incorporation into the inhibited enzyme.

The inhibited pepsin C (130mg) was fragmented by dissolving it in 15ml of 0.02M-hydrochloric acid and adding native pepsin (7mg). The incubation was continued at 37°C for 40h at which time the trace of insoluble material left was centrifuged off. Scintillation counting revealed that 95% of the radioactivity was in the soluble fractions.

The supernatant was fractionated on a column of Bio-Gel P6, equilibrated with 5% acetic acid (Fig. 6). The radioactivity was eluted in two major peaks, A and B. Fraction A contained 35% of the

Fig. 4. Inactivation of pepsin C (1.2mg/ml) by diazoacetyl-DL-norleucine methyl ester (1.2mM) and the effect of acetyl-L-phenylalanyl-L-di-iodotyrosine on the inactivation. ○, Without acetyl-L-phenylalanyl-L-di-iodotyrosine; ●, with acetyl-L-phenylalanyl-L-di-iodotyrosine (3mM).

Fig. 5. Inactivation of a large batch of pepsin C (1.2mg/ml) by diazoacetyl-DL-norleucine methyl ester (1.2mM) in the presence of cupric ions (0.9mM) in 0.2M-acetate buffer, pH 5.0, at 20°C.

Fig. 6. Chromatography of the peptic digest of inhibited pepsin C on a column (3 cm $\times$ 52 cm) of Bio-Gel P6, equilibrated with 5% acetic acid. Fractions of volume 5 ml were collected and 0.2 ml samples were taken for scintillation counting. $\circ$, E_{280} ; $\bullet$, radioactivity.

total radioactivity and because of its elution volume, was assumed to be undigested material. Fraction B contained 40% of the total radioactivity. These fractions were pooled and dried *in vacuo*. The residue was redissolved in a small volume of 5% acetic acid and Fig. 7 shows the elution pattern obtained after rechromatography on Bio-Gel P4. No material absorbing at 280 nm was present. The fractions containing the label were pooled and concentrated and subjected to electrophoresis at pH 3.6 on Whatman no. 1 paper. Scanning the paper revealed that the radioactive material was neutral at this pH (Fig. 8) as it migrated to a position identical with that of a fructose marker. This radioactive band contained 33% of the total radioactivity incorporated into the enzyme. The band was cut out, stitched into a fresh sheet of paper and subjected to electrophoresis at pH 2.0. This gave a partial separation of the radioactive material into two bands, B1 (at 15 cm towards the cathode) and B2 (at 12 cm towards the cathode). The recoveries were 18 and 14% respectively of the total radioactivity incorporated. These bands were cut out and the material was concentrated on the paper by folding the paper along the long axis of the band into a kind of tent and allowing 5% acetic acid to diffuse up to the apex. The strips were dried and stitched

Fig. 7. Rechromatography of fraction B on a column (2 cm $\times$ 50 cm) of Bio-Gel P4 equilibrated with 5% acetic acid. Fractions of volume 2 ml were collected and 0.1 ml samples were taken for counting of radioactivity.

into fresh sheets of paper and subjected to descending chromatography in 2-methylpropan-2-ol-butan-2-one-water (2:2:1, by vol.). Peptide B1 had an R_F 0.53 whereas peptide B2 had an R_F 0.36. The recoveries were 5% (0.15 μ mol) and 3% (0.1 μ mol) respectively of the total radioactivity incorporated into the protein. The material in these two bands

was eluted from the papers with 5% acetic acid and dried *in vacuo*.

Table 3 shows the amino acid analyses obtained from hydrolysed samples of peptide B1 and peptide B2. The composition of the peptide B1 is (Asp,Thr,-Val,Ile,Nle*), the norleucine being derived from the inhibitor. The total amount of norleucine in the hydrolysate of peptide B1 was in excellent agreement with the $0.15\mu\text{mol}$ calculated from the radioactivity recovery.

Dansyl-Edman degradation showed that the *N*-terminal sequence of peptide B1 was Ile-Val. Treatment of peptide B1 with carboxypeptidase A released $0.93\mu\text{mol}$ of threonine/ μmol of peptide and nothing else so that peptide B1 must be Ile-Val-Asp-Thr with inhibitor.

Table 2 shows that peptide B2 contained proline, leucine, alanine and phenylalanine in non-integral amounts but dansyl-Edman degradation revealed a homogeneous *N*-terminal sequence of Ile-Val-Asp-. It is highly likely that this peptide overlaps with the Ile-Val-Asp-Thr sequence, but the low yield of material prohibited determination of the precise sequence.

A sample of peptide B1 was treated on paper

* Abbreviation: Nle (in sequences), norleucine.

Fig. 8. Radiochromatogram of fraction B after electrophoresis at pH 3.6 (40 V/cm; 2 h).

with triethylamine by allowing this to diffuse in from the edges of the band as in wetting a paper for electrophoresis. The paper was then subjected to electrophoresis at pH 3.6 (40 V/cm, 2 h). The untreated peptide migrated 5 cm towards the cathode in accord with its supposed neutral composition. The treated peptide (detected by ninhydrin) migrated 0.5 cm towards the cathode. No radioactivity could be detected anywhere on this strip, presumably because the hydroxyacetylnorleucine assumed to be released was soluble in the 'white spirit' in the electrophoresis tanks.

DISCUSSION

The demonstration that diazoacetyl-DL-norleucine methyl ester inactivates pepsin C with a concomitant incorporation of the inhibitor into a specific residue on the enzyme suggests that the reaction may be recurring at a group involved in the catalytic activity. It is not possible to state categorically that the group being modified is part of the active site but since the competitive inhibitor, acetyl-L-phenylalanyl-L-di-iodotyrosine, protected the enzyme against inactivation to some extent and since very little inhibitor was incorporated into denatured pepsin C, the indications are that the reaction is occurring at the active site. It is difficult, on this basis, to rationalize the results observed with pepsinogen C which, as the inactive precursor, ought to have its active site masked or at least, not in the native configuration. The fact that the zymogen lost 65% of its potential activity on incubation at pH 5 in contrast with a 10% loss at pH 7 might be explained if the zymogen has a conformation at pH 7 which does not have the active site in its native form, whereas at pH 5, the molecule begins to refold so that the active site is beginning to exist in the native form but without release of the activation peptides (which requires a lower pH) to form the active enzyme. Thus the activation peptides could protect the active site to some extent so that the zymogen only undergoes a partial inactivation under conditions which fully inactivate the enzyme. This hypothesis obviously requires further study.

It appears, from the following arguments, that

Table 3. Amino acid analyses of the labelled peptides

Hydrolysis was carried out *in vacuo* at $105\pm 2^\circ\text{C}$ for either 24 h or 72 h. Values are calculated relative to norleucine 1.0.

	Asp	Thr	Ser	Pro	Gly	Ala	Val	Ile	Leu	Nle	Phe
Peptide B1 72 h	1.0	1.0*	—	—	—	—	1.0	0.9	—	1.0	—
Peptide B2 24 h	1.0	1.8	1.0	0.7	1.0	0.5	0.9	0.7	0.7	1.0	0.3

* Corrected to zero-time hydrolysis.

the site of attachment of the inhibitor is the β -carboxyl group of an aspartate residue: (a) the labelled peptides are neutral (from their migration on electrophoresis); (b) the base-lability of the enzyme-inhibitor bond suggests an ester bond; (c) the release of the inhibitor residue from the labelled peptide produces a peptide with a partial negative charge on electrophoresis at pH 3.6.

Thus, the active site peptide, Ile-Val-Asp-Thr, identified for pepsin C is identical with part of the active-site peptide, Ile-Val-Asp-Thr-Gly-Thr-Ser, isolated from porcine pepsin by Bayliss *et al.* (1969). The homology is noteworthy in view of the fact that, although the two enzymes have similar specificities with regard to the amino acids involved in the bonds split (Ryle & Porter, 1959), pepsin has activity against some peptide substrates which pepsin C does not attack. The difference in activity cannot be explained by a different amino acid sequence around (one of) the reactive carboxyl group(s). The inability of the minor enzyme to hydrolyse small peptide substrates could be due to a number of factors, e.g. it could be that, analogously with lysozyme, the enzyme may require the substrate to have a long chain of amino acid residues before it can carry out the hydrolysis.

In view of the homologies found between trypsin, chymotrypsin and elastase with regard to their amino acid sequences, active sites and over-all conformations (Shotton & Hartley, 1970) it is not surprising that two similar enzymes like pepsin and pepsin C should have identical sequences around (one of) the reactive carboxyl residue(s).

This work was supported by a grant from the Medical Research Council and J.K. acknowledges the receipt of a Scholarship for Training in Research Methods from the Medical Research Council.

REFERENCES

- Ambler, R. P. (1967). In *Methods in Enzymology*, vol. 11, p. 346. Ed. by Hirs, C. H. W. New York: Academic Press Inc.
- Anson, M. L. (1948). In *Crystalline Enzymes*, p. 305. Ed. by Northrop, J. H., Kunitz, M. & Herriott, R. M. New York: Columbia University Press.
- Bayliss, R. S., Knowles, J. R. & Wybrandt, G. B. (1969). *Biochem. J.* **113**, 377.
- Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943). *Biochem. J.* **37**, 354.
- Clement, G. E. & Snyder, S. (1966). *J. Am. chem. Soc.* **88**, 5338.
- Cornish-Bowden, A. J. & Knowles, J. R. (1969). *Biochem. J.* **113**, 353.
- Denburg, J. L., Nelson, R. & Silver, M. S. (1968). *J. Am. chem. Soc.* **90**, 479.
- Fry, K. T., Kim, O. K., Spona, J. & Hamilton, G. A. (1968). *Biochem. biophys. Res. Commun.* **30**, 489.
- Gray, W. R. (1967). In *Methods in Enzymology*, vol. 11, p. 469. Ed. by Hirs, C. H. W. New York: Academic Press Inc.
- Lee, D. & Ryle, A. P. (1967). *Biochem. J.* **104**, 742.
- Lundblad, R. L. & Stein, W. H. (1969). *J. biol. Chem.* **244**, 154.
- Michl, H. (1951). *Mh. Chem.* **82**, 489.
- Morse, D. & Horecker, B. L. (1966). *Analyt. Biochem.* **14**, 429.
- Porter, R. R. (1957). In *Methods in Enzymology*, vol. 4, p. 221. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Rajagopalan, T. G., Moore, S. & Stein, W. H. (1966b). *J. biol. Chem.* **241**, 4940.
- Rajagopalan, T. G., Stein, W. H. & Moore, S. (1966a). *J. biol. Chem.* **241**, 4295.
- Ryle, A. P. (1960). *Biochem. J.* **75**, 145.
- Ryle, A. P. (1965). *Biochem. J.* **96**, 6.
- Ryle, A. P. (1970). In *Methods in Enzymology*, vol. 19, p. 316. Ed. by Perlmann, G. E. & Lorand, L. New York: Academic Press Inc.
- Ryle, A. P. & Hamilton, M. P. (1966). *Biochem. J.* **101**, 176.
- Ryle, A. P., Leclerc, J. R. & Falla, F. (1968). *Biochem. J.* **110**, 4P.
- Ryle, A. P. & Porter, R. R. (1959). *Biochem. J.* **73**, 75.
- Shotton, D. M. & Hartley, B. S. (1970). *Nature, Lond.*, **225**, 802.
- Smithies, O. (1955). *Biochem. J.* **61**, 629.
- Spackman, D. H. (1963). *Fedn Proc. Fedn Am. Socs exp. Biol.* **22**, 244.
- Stepanov, V. M. & Vaganova, T. I. (1968). *Biochem. biophys. Res. Commun.* **31**, 825.
- Woods, K. R. & Wang, K.-T. (1967). *Biochim. biophys. Acta*, **133**, 369.
- Zeffren, E. & Kaiser, E. T. (1967). *J. Am. chem. Soc.* **89**, 4204.
- Zollinger, H. (1961). *Diazo and Azo Chemistry*, p. 164. New York: Interscience Publishers Inc.