Bovine Pepsinogens and Pepsins

THE SEQUENCE AROUND A REACTIVE ASPARTYL RESIDUE

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The specific inhibitor, *N*-diazooacetyl-norleucine methyl ester reacts stoichiometrically with bovine pepsin resulting in a simultaneous loss of all enzymic activity. A peptide containing a modified aspartyl group was isolated from bovine pepsin labelled with ¹⁴C-labelled inhibitor. The aspartic acid residue is presumed to be part of the active centre and is in the same heptapeptide sequence as in porcine pepsin: Ile-Val-Asp-Thr-Gly-Thr-Ser.

Porcine pepsin has at least two carboxyl groups that are involved in the mechanism of action of the enzyme (Cornish-Bowden & Knowles, 1969; Lundblad & Stein, 1969). Substitution of one carboxyl group irreversibly destroys the activity, whereas substitution of the other interferes stereochemically with substrate binding (Erlanger, Vratsanos, Wassermann & Cooper, 1967). A variety of diazo compounds has been used to label the first carboxyl group (Herriott, 1947; Rajagopalan, Stein & Moore, 1966; Delpierre & Fruton, 1965, 1966; Ong & Perlmann, 1967; Bayliss & Knowles, 1968; Fry, Kim, Spona & Hamilton, 1968; Badley & Teale, 1968; Ginodman, Valueva, Kozlov & Shkarenkova, 1969). Peptides containing this carboxyl group after modification have been isolated and their sequences have been determined (Knowles & Wybrandt, 1968; Stepanov & Vaganova, 1968; Bayliss, Knowles & Wybrandt, 1969; Fry, Kim, Spona & Hamilton, 1970). The sequence around this residue in porcine pepsin (determined independently by all these workers, completely or in part) is:

Ile-Val-Asp-Thr-Gly-Thr-Ser-Leu

Kay & Ryle (1971) have found part of the same sequence (Ile-Val-Asp-Thr) for porcine pepsin C. In each case the diazo compound esterified the pepsin in a 1:1 stoichiometric reaction in the presence of Cu²⁺ ions, with the concomitant loss of all proteolytic activity. It has been suggested that the β -carboxyl group of this esterified aspartic acid residue is at the active site of pepsin (e.g. see Rajagopalan *et al.* 1966).

Previous studies from this laboratory have demonstrated that bovine and porcine pepsinogens and pepsins have several very similar characteristics, yet differ in a number of features. For example, the proteins of both species have a large number of acidic and small number of basic residues,

but they differ considerably in their content of certain individual amino acid residues (Chow & Kassell, 1968; Lang & Kassell, 1971). The two enzymes have similar pH optima and are qualitatively similar in their specificities (Fruton & Bergmann, 1940). Quantitatively, they differ in their relative reactivity with various substrates (Lang & Kassell, 1971), bovine pepsin hydrolysing the substrates tested less efficiently than porcine pepsin.

Because of the qualitative similarities, but quantitative differences in composition and specificity, it was decided to determine whether bovine pepsin has a similar reactive aspartic acid residue. This paper describes the reaction of bovine pepsin with a ¹⁴C-labelled specific inhibitor, *N*-diazooacetyl-norleucine methyl ester [the inhibitor developed by Rajagopalan *et al.* (1966) for porcine pepsin], and the identification of a heptapeptide containing the reactive aspartic acid residue.

MATERIALS

N-Diazooacetyl-DL-[1-¹⁴C]norleucine methyl ester. Chromatographically pure DL-[1-¹⁴C]norleucine (specific radioactivity 52.7 mCi/mmol) was obtained from International Chemical and Nuclear Corp., Irvine, Calif., U.S.A. The starting material for the synthesis was prepared by diluting this compound in solution with unlabelled DL-norleucine to give a specific radioactivity of 90 μ Ci/mmol.

Glycyl[1-¹⁴C]norleucine was prepared by treating DL-[1-¹⁴C]norleucine with chloroacetic anhydride (Birnbaum, Levintow, Kingsley & Greenstein, 1952) followed by amination of the chloroacetyl derivative (Rao, Birnbaum, Kingsley & Greenstein, 1952). Paper chromatography in the system: butan-1-ol-acetic acid-water (4:1:5, by vol.) gave a single ninhydrin-positive spot corresponding to authentic glycyl-DL-norleucine. The glycyl-DL-norleucine was converted into the methyl ester hydrochloride by the procedure of Brenner & Huber (1953). *N*-Diazooacetyl-DL-[1-¹⁴C]norleucine methyl ester was prepared from glycyl[1-¹⁴C]norleucine methyl ester by the method

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of Rajagopalan *et al.* (1966). The overall yield from [$1\text{-}^{14}\text{C}$]norleucine was usually about 10%. The *N*-diazoacetylnorleucine methyl ester is unstable and light-sensitive. Since the immediate precursor, glycylnorleucine methyl ester hydrochloride, is stable, the amount needed was diazotized just before use.

Enzymes. Bovine pepsinogen was prepared by the method of Chow & Kassell (1968). The pepsin was prepared from the pepsinogen as described by Kassell & Meitner (1970).

Crystalline porcine pepsin (EC 3.4.4.1) was obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Carboxypeptidase A (EC 3.4.2.1) (Worthington Biochemical Corp.) was treated with di-isopropyl phosphorfluoridate before use.

Other chemicals. Solvents for chromatography and electrophoresis were prepared from redistilled A.R.-grade reagents. Reagents for sequence determination were purified as described by Edman & Begg (1967). All other reagents and apparatus have been described previously (Meitner & Kassell, 1971).

METHODS

Scintillation counting. The scintillation fluid was toluene (reagent grade) containing 2,5-diphenyloxazole (5g/l) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (200mg/l). It was stored in a dark bottle at 4°C.

Aqueous samples (50 μ l) for radioactivity counting were dissolved completely in 0.5 ml of NCS solubilizer (Amersham-Searle, Des Plaines, Ill., U.S.A.) by vigorous agitation of the counting bottle until no more schlieren lines could be seen. Scintillation fluid (10 ml) was then added, and after mixing, the bottles were equilibrated in the Tri-Carb liquid-scintillation spectrometer (model 3950; Packard Instrument Co., Downers Grove, Ill., U.S.A.) for 30 min before the radioactivity was counted. The counting efficiency was determined periodically with an internal standard of [^{14}C]toluene (Packard Instrument Co.) and was always better than 83%.

Location of radioactive compounds on paper. Radioactive areas on chromatograms and electrophoretograms were located with a gas-flow strip scanner (Actigraph III; Nuclear-Chicago Corp., Des Plaines, Ill., U.S.A.). In preparative experiments, guide strips were cut longitudinally in the direction of solvent flow or electrophoresis. These strips were first scanned for radioactivity and then a sample strip was dipped in the ninhydrin-cadmium reagent of Heilmann, Barrolier & Watzke (1957). The areas corresponding to isotopically labelled peptides were cut from the papers and eluted with water.

Separation of peptides on paper. Whatman 3MM paper was washed with 0.1M-HCl, water and 95% (v/v) ethanol before use. Electrophoreses were performed on a flat-plate electrophoresis apparatus (Savant Instruments, Hicksville, N.Y., U.S.A.) with Whatman 3MM paper. The conditions used for electrophoresis and chromatography were similar to those described by Bayliss *et al.* (1969): electrophoresis at pH 3.5 was for 2.5 h at 162 V/cm in pyridine-acetic acid-water (1:10:89, by vol.); electrophoresis at pH 1.9 was for 2 h at 146 V/cm in aq. 9% (w/v) formic acid; descending chromatography was carried out for 4 h in 2-methylpropan-2-ol-butan-2-one-water (2:2:1, by vol.).

Analytical methods. Amino acid analyses were performed on a Beckman-Spinco model 120B amino acid analyser. The Edman degradation of the peptide was carried out by a manual modification (Blombäck, Blombäck, Edman & Hessel, 1966) of the method of Edman & Begg (1967). This is a three-stage method: formation of the phenylthiocarbamoyl derivative, cyclization to the thiazolinone and conversion into the phenylthiohydantoin. The cyclization was carried out twice at each step. The phenylthiohydantoin derivatives were identified by t.l.c. on silica-gel plates (Edman & Sjöquist, 1956; Brenner, Niederwieser & Pataki, 1969). Carboxypeptidase A digestion was used under conditions similar to those used by Fry *et al.* (1970).

Enzyme assay. Pepsin was assayed by a modification of the haemoglobin method of Anson (1939) and by its milk-clotting ability. Both procedures have been described previously (Chow & Kassell, 1968).

RESULTS

Inhibition of pepsin with *N*-diazoacetyl[$1\text{-}^{14}\text{C}$]norleucine methyl ester. Bovine pepsin was inhibited by the same method used for porcine pepsin (Rajagopalan *et al.* 1966). A preliminary experiment demonstrated that the loss of activity measured by milk clotting and by haemoglobin digestion occurred at the same rate and was complete in 20 min and that 1 mol of norleucine was incorporated/mol of the bovine pepsin, as measured by radioactivity and by amino acid analysis. No non-specific labelling occurred. Bovine pepsin (320 mg), freshly prepared from pepsinogen, was dissolved in 0.042M-sodium acetate, pH 5, to give a final protein concentration of 2 mg of protein/ml. The solution was maintained at 14°C with stirring. Cupric acetate (0.1M; 3 ml) was added and the solution was incubated for 10 min. During this time no loss of enzyme activity was detected by the milk-clotting test. After 10 min a methanolic solution of *N*-diazoacetyl[$1\text{-}^{14}\text{C}$]norleucine methyl ester (83 mg in 3.2 ml) was added. The progress of the inhibition was followed by observing the disappearance of milk-clotting ability of the pepsin. After 20 min the pepsin was 99.7% inhibited. A small amount of brown precipitate was removed by centrifugation, and the clear reaction mixture (150 ml) was applied to a desalting column (4.6 cm $\times$ 45 cm) of Sephadex G-25 and eluted with water (Bayliss *et al.* 1969). The pooled protein peak contained 255 mg of inhibited pepsin.

Digestion of the inhibited pepsin and isolation of a ^{14}C -labelled peptide. The peptic digestion and the isolation of the peptide were conducted according to the procedure of Bayliss *et al.* (1969). The elution pattern of the digest on Sephadex G-25 is shown in Fig. 1. Peak A was pooled as indicated and contained 3.3 μ mol of radioactive peptides, calculated on the basis of radioactivity (about 55%

of the radioactivity eluted from the column). Three further steps: electrophoresis at pH 3.5, paper chromatography and electrophoresis at pH 1.9, in this order, yielded 0.5 μ mol of a peptide that gave a single radioactive ninhydrin-positive spot. The procedure was repeated with another 320 mg of pepsin with the same results. The total amount of peptide available for analysis was thus 1 μ mol.

Analysis of the peptide. A portion of the peptide was hydrolysed *in vacuo* with 5.7M-hydrochloric acid for 24 h at 110°C. The amino acid content is shown in Table 1.

The peptide (0.4 μ mol based on the amino acid analysis) was carried through five steps of the Edman degradation with the results shown in Table 2. Although the yields were rather low, sufficient phenylthiohydantoin derivative of amino acid was

available for unequivocal identification at each step in two t.l.c. systems. The *N*-terminal sequence, Ile-Val-Asp-Thr-Gly-, was established in this way. Carboxypeptidase A digestion (Table 3) showed that the *C*-terminal sequence was -Gly-Thr-Ser. Therefore the sequence of the heptapeptide is:

DISCUSSION

The pig and the cow are fairly closely related species, but their pepsinogens and pepsins have numerous differences in amino acid composition (Chow & Kassell, 1968; Lang & Kassell, 1971). The fact that the sequences surrounding the reactive aspartic acid are identical in the two pepsins supports earlier suggestions, based on reactions with inhibitors, that this is the catalytic site, an area of the molecule in which a precise structure is required and evolutionary changes are not permissible. Similarly, although no functional relationship would be expected between an acid protease of a simple mould and a gastric enzyme, the isolation of a related peptide with a reactive aspartic acid residue

Fig. 1. Elution of the 12 h pepsin digest of inhibited pepsin from Sephadex G-25 with water at room temperature. The column (1.9 cm $\times$ 150 cm) had a void volume ($\downarrow$) of 128 ml. Fractions (2.6 ml) were collected at a flow rate of 14 ml/h. $\circ$, E_{220} ; $\square$, radioactivity. The fractions were pooled as indicated by the bar.

Table 2. Edman degradation of the peptide

Solvent D is from Edman & Sjöquist (1956). Solvents II and III are from Brenner *et al.* (1969). The yield was determined from E_{269} ; with threonine the abnormal shape of the peak increases the apparent yield. No other phenylthiohydantoin (PTH) derivatives of amino acids were present in significant amounts.

Step	PTH-amino acid	Yield (mol)	T.l.c. systems
1	Ile	0.59	D and II
2	Val	0.41	D and II
3	Asp	0.23	II and III
4	Thr	<0.21	II and III
5	Gly	0.04	II and III

Table 1. Amino acid composition of the radioactive peptide

The number of residues was calculated on the basis of 1 residue of aspartic acid/molecule. Uncorrected values for a 24 h hydrolysis of the peptide are reported. No other amino acid residues were present in significant amounts.

Amino acid	Residues/molecule	
	Found	Nearest whole no.
Asp	1.0	1
Thr	1.82	2
Ser	1.04	1
Gly	1.13	1
Val	0.73	1
Ile	0.65	1
Norleucine	0.84	1

Table 3. Digestion of the peptide with carboxypeptidase A

Digestion was at 37°C with 0.102 μ mol of peptide and 0.1 mg of carboxypeptidase A in 1.1 ml of 20 mM- NH_4HCO_3 buffer, pH 8.2. No other amino acids were present in significant amounts above the blank values due to the enzyme.

Digestion time (h)	Mol of amino acid/mol of peptide		
	Ser	Thr	Gly
6	0.46	0.17	0.04
17	0.77	0.44	0.16

(Ile-Ala-Asp-Thr-Gly-Thr-Thr) from the penicillopepsin of *Penicillium janthinellum* (Sodek & Hofmann, 1970) implies a biochemical relationship between the two and is an even more striking indication of the constancy of the active centre. Acidic proteases thus constitute a third homologous series of proteolytic enzymes, comparable with the serine and the thiol proteases.

Kinetic comparisons of bovine and porcine pepsins (Lang & Kassell, 1971) showed that the K_m values with the synthetic substrates tested were the same, within experimental error, for the two enzymes, although the k_{cat} values differed. Thus it is probable that the specificity sites of the two enzymes also have similar structures, and that the differences accounting for the variations in k_{cat} will be found in compositional or conformational changes in the other portions of the peptide chain that form part of the catalytic site.

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