Inhibition of pepsin by analogues of pepsinogen-(1–12)-peptide with substitutions in the 4–7 sequence region

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Derivatives of the 1–12 sequence of pig pepsinogen were prepared by solid-phase peptide synthesis. The three derivatives contain substitutions in the 4–7 region of the 1–12 sequence. Glycine was used to replace the hydrophobic residues -Val-Pro-Leu-Val- in pairs. After cleavage and purification, the synthetic peptides were compared with a synthetic peptide of the native sequence, prepared at the same time, with respect to their ability to inhibit the pepsin-catalysed clotting of milk. Inhibitory potency, determined from plots of percentage inhibition versus concentration of synthetic peptide, is inversely correlated with the substitution of glycine residues for the hydrophobic residues. Therefore the equilibrium inhibition of pepsin by these peptides is dominated by the hydrophobic nature of the 4–7 sequence region.

Several small peptide fragments released on activation of pig pepsinogen are inhibitors of pepsin thus generated (Herriott, 1939, 1941). Previous publications from this laboratory have demonstrated that pepsinogen-(1–16)-peptide is the strongest inhibitory peptide (Dunn *et al.*, 1978; Dunn & Deyrup, 1979):

Leu-Val-Lys-Val-Pro-Leu-Val-Arg-Lys-Lys-Ser-Leu-Arg-Gln-Asn-Leu

We have also shown that shorter analogues, such as pepsinogen-(1–12)-peptide, will inhibit nearly as well as pepsinogen-(1–16)-peptide. By using solid-phase peptide synthesis combined with chemical modification, we have obtained evidence that the positively charged residues are not essential to the inhibitory potency of the peptides. Such evidence can make the synthesis of effective analogues smoother, since each complex amino acid adds to the possibility of sequence errors, decreases the yield and

increases the cost of synthesis. The only area of pepsinogen-(1–16)-peptide that has not been probed by synthesis or modification is the central block of hydrophobic residues in positions 4–7 (-Val-Pro-Leu-Val-). In the present paper we detail the synthesis, purification and evaluation of three analogues of the pepsinogen-(1–12)-peptide

sequence where glycine has replaced residues 4 and 5, 6 and 7 and 4–7 (II–IV). We have used the pepsinogen-(1–12)-peptide sequence as a control, since it is nearly as good as pepsinogen-(1–16)-peptide as an inhibitor of pig pepsin, and elimination of arginine-13, glutamine-14 and asparagine-15 removes three expensive and potentially troublesome residues from the synthetic programme. In these analogues, the charged residues at positions 3, 8, 9 and 10 are retained, since they serve to make the resulting peptides highly water-soluble. This facilitates purification and evaluation. A preliminary

Abbreviations used: pepsinogen-(1–16)-peptide, the peptide corresponding to the *N*-terminal 16 residues of pepsinogen; likewise other peptides are named as pepsinogen-(*X*–*Y*)-peptide, where *X* and *Y* are numbers of the amino acids in the pepsinogen numbering scheme; synthetic variants are named in accordance with standard definitions (IUPAC–IUB Commission on Biochemical Nomenclature, 1967); all amino acids were of the *L*-configuration; Boc, *t*-butoxycarbonyl; Cbz, benzylloxycarbonyl; h.p.l.c.; high-pressure liquid chromatography.

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Leu-Val-Lys-Val-Pro-Leu-Val-Arg-Lys-Lys-Ser-Leu
(I) Native sequence pepsinogen-(1–12)-peptide

Leu-Val-Lys-Gly-Gly-Leu-Val-Arg-Lys-Lys-Ser-Leu
(II) [Gly^{4,5}]pepsinogen-(1–12)-peptide

Leu-Val-Lys-Val-Pro-Gly-Gly-Arg-Lys-Lys-Ser-Leu
(III) [Gly^{6,7}]pepsinogen-(1–12)-peptide

Leu-Val-Lys-Gly-Gly-Gly-Gly-Arg-Lys-Lys-Ser-Leu
(IV) [Gly^{4–7}]pepsinogen-(1–12)-peptide

account of some of this work has been presented (Dunn & Lewitt, 1980).

Experimental

Peptide synthesis

All peptides were synthesized by stepwise solid-phase procedures (Barany & Merrifield, 1979), manual methods being used for washing and reagent addition. α -Boc-protected amino acids were obtained from Vega-Fox Biochemicals (Tucson, AZ, U.S.A.). Trifluoroacetic acid was from Eastman (Rochester, NY, U.S.A.) and was redistilled before use. Bio-Beads (1% cross-linked polystyrene with 0.69 mequiv. of chloromethyl substitution/g of dry resin) were obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.)

The general strategy for the synthesis is shown in Scheme 1. Attachment of Boc-leucine was accomplished by the caesium salt method (Gisin, 1973) with a substitution level of 40.6% of theoretical.

A 5 g portion of Boc-leucine-resin was placed in a glass reaction vessel with coarse sintered-glass filter and stopcock at one end and a 14/20 addition port at the other end. This was placed in a reciprocating shaker. Sequential synthesis was performed with the use of 25% (v/v) trifluoroacetic acid in dichloromethane for deprotection and a 10% (v/v) solution of either ethyldi-isopropylamine or triethylamine in

dichloromethane for neutralization. The protected amino acid (3-fold excess over resin amino groups) to be added and an equivalent amount of 1-hydroxybenzotriazole hydrate were dissolved in 10 ml of dichloromethane. In some cases a few drops of dry dimethylformamide were added to dissolve the protected amino acid before the dichloromethane was added. An equimolar amount of dicyclohexylcarbodi-imide dissolved in dichloromethane was used and the reaction time was 120 min.

After coupling and washing steps, approx. 2 mg of the wet resin was removed with a small spatula and placed in a small plastic centrifuge tube with a snap top. Two drops of 10% (v/v) ethyldi-isopropylamine in dichloromethane were added to the tube followed by 0.5 ml of a solution of fluorescamine (5 mg in 3 ml of acetone) (Felix & Jimenez, 1973) and shaken for a few minutes manually. The resin beads were then transferred to a 13 mm paper disc in a Hirsch funnel with a glass transfer pipette. The resin was then washed with dichloromethane followed by 100% ethanol. The filter paper was picked up with tweezers and placed under a fluorescent light. The presence of an intense fluorescence indicated an incomplete coupling reaction. The absence of fluorescence was taken to indicate greater than 99.5% coupling. In most cases during these syntheses, a single coupling was sufficient to yield a negative bead test as above.

Scheme 1. Synthetic strategy for preparation of precursors for peptides I-IV
For full experimental details see the text.

For the cases where the bead test was positive, a second coupling was performed, after which, in all cases, a negative bead test was then obtained. The protected lysine was supplied as a *t*-butylammonium salt and was converted into the free acid by ethyl acetate extraction from 0.5 M-H₂SO₄ solution.

As indicated in Scheme 1, all the peptides prepared share a common 8–12 sequence. Accordingly, a single four-step synthesis was performed to give protected pepsinogen-(8–12)-peptide-resin. This was split into two portions. The first half was coupled with Boc-valine followed by Boc-leucine to yield protected pepsinogen-(6–12)-peptide-resin. This was then split into two subportions. The first was subjected to five additional steps to yield protected pepsinogen-(1–12)-peptide-resin with the native sequence. The second subportion was coupled with Boc-glycine followed by Boc-glycine again, and then Boc-lysine, Boc-valine and Boc-leucine in succession. This yields protected [Gly^{4,5}]pepsinogen-(1–12)-peptide-resin. The second portion of protected pepsinogen-(8–12)-peptide-resin was coupled with Boc-glycine followed by Boc-glycine again to yield protected [Gly^{6,7}]pepsinogen-(6–12)-peptide-resin. This was split into two subportions. The first subportion was coupled with Boc-proline, Boc-valine, Boc-lysine, Boc-valine and Boc-leucine in succession to yield protected [Gly^{6,7}]pepsinogen-(1–12)-peptide-resin. The final subportion was coupled with Boc-glycine, Boc-glycine, Boc-lysine, Boc-valine and Boc-leucine in succession to yield protected [Gly^{4–7}]pepsinogen-(1–12)-peptide-resin.

Solid-phase sequencing

At several points during the syntheses, and in all cases at the end of the syntheses, solid-phase peptide sequencing (Matsueda *et al.*, 1981) was performed directly on 10 mg samples of peptidyl-resin (M. Horn & A. Bonner, personal communication). The sample was taken after the methanol wash following coupling. This dried the resin and facilitated weighing and transferring. The small sample of synthetic peptidyl-resin was mixed with 1 g of silylated glass beads, and this mixture was placed in a 15 cm column in a Sequemat Mini-15 automatic sequencer. Sufficient cycles of the Edman degradation were performed to sequence to the end of the peptide. The resulting products were converted into amino acid phenylthiohydantoin derivatives by drying the samples, adding 200 μ l of aq. 20% (v/v) trifluoroacetic acid, heating the mixture for 15 min at 85°C and extracting it with ethyl acetate. In later experiments a Sequemat P-6 Auto Converter was used. These extracts were evaporated to dryness and taken up in 100 μ l of ethyl acetate. Then 2–20 μ l portions of those solutions were spotted on 20 cm $\times$ 20 cm plates of silica gel 60 (0.25 mm thickness, F-254; EM

Laboratories) and developed with chloroform/ethanol (49:1, v/v). In each case the results confirmed the correctness of the synthetic procedure. Because of the unique sequences prepared, the appearance of preview spots was readily seen. In all cases, visual observation of the resulting t.l.c. plates under u.v. light revealed only a minor amount (less than 2% estimated) of preview. A more quantitative measure can be obtained by h.p.l.c. analysis of the product of the first degradation cycle. In all cases the sequencing results agreed with the fluorescence bead tests.

HF cleavage

The completed peptides were removed from the polystyrene resin, and all side-chain-protecting groups were removed by treatment with anhydrous HF in a Toho Kasei liquid-HF apparatus. For this 10 ml of liquid HF and 1 ml of redistilled anisole per g of peptidyl-resin were used. The peptidyl-resin/anisole was cooled in an ethanol/solid CO₂ bath while HF was distilled into the reaction vessel, and then the sample was allowed to come to 0°C in an ice bath. A 30 min period was allowed for the temperature change, and then the sample was stirred magnetically for 1 h. The HF was removed with a water aspirator and an N₂ stream. Anisole was extracted by several ethyl acetate washes, and the sample was transferred to a coarse sintered-glass funnel. The crude peptide was then extracted by stirring the resin beads with three portions of aq. 50% (v/v) acetic acid. The acetic acid was removed by rotary evaporation, water was added, and the sample was freeze-dried.

Purification of synthetic peptides

All four crude peptide samples were dissolved in 0.01 M-ammonium acetate buffer, pH 5.0. The conductivity of the buffer alone was 1.03 mS. The peptide solutions were diluted if necessary to give conductivities below 4 mS. The samples were run on 0.9 cm $\times$ 40 cm columns of CM-Sephacrose equilibrated with the 0.01 M-ammonium acetate buffer. After the total sample was applied and an additional 50 ml of starting buffer was run through, a linear gradient of 0.01–0.6 M-ammonium acetate buffer, pH 5.0, was begun to elute the basic peptides. The conductivity of the 0.6 M-ammonium acetate was 37.6 mS. Portions (50 μ l) of the 2 ml fractions were analysed by adding 0.8 ml of 0.1 M-phosphate buffer, pH 8, and 250 μ l of a solution of 5 mg of fluorescamine in 20 ml of acetone with rapid vortex-mixing. The fluorescence intensities were read on an Aminco Bowman Spectrophotofluorometer with excitation at 390 nm and emission at 480 nm. The resulting data are plotted in Fig. 1 for all four samples. Fractions were pooled, freeze-dried and redissolved in a measured volume of water, and amino acid analyses

Fig. 1. Ion-exchange chromatography of crude synthetic peptides on CM-Sephacrose in ammonium acetate buffer, pH 5.0. For full experimental details see the text. (a) Separation of synthetic pepsinogen-(1-12)-peptide; (b) separation of synthetic [Gly^{4,5}]pepsinogen-(1-12)-peptide; (c) separation of synthetic [Gly^{6,7}]pepsinogen-(1-12)-peptide; (d) separation of synthetic [Gly⁴⁻⁷]pepsinogen-(1-12)-peptide. ●—●, Conductivity readings of the eluate arising from the gradient passing from 0.01M-ammonium acetate to 0.6M-ammonium acetate; —, fluorescence intensity in arbitrary units obtained by treating 50 μ l portions of the 2 ml fractions with fluorescamine. The black horizontal bar in each panel marks the fractions that were pooled, analysed for amino acid composition, assayed for inhibitory activity and subjected to further purification by h.p.l.c.

were performed on small portions of each pool. The analyses of the best pools (indicated by the bars in Fig. 2) for the separation of each peptide are presented in Table 1(a). These peaks give ratios closest to the theoretical values and were employed for further study.

H.p.l.c. purification of peptides

To provide further purification of these samples we employed a Waters High Performance Liquid Chromatograph consisting of two model 6000A solvent-delivery systems, a model 660 solvent programmer, model 440 Absorbance Detector and model 450 Variable Wavelength Absorbance Detector, U6K injector and μ Bondapak C₁₈ reverse-phase column. Solvent A was aq. 10% (v/v) methanol containing 5 mM-trifluoroacetic acid, solvent B was aq. 90% (v/v) methanol containing 5 mM-trifluoroacetic acid, and solvent C was aq. 25% (v/v) methanol containing 5 mM-trifluoroacetic acid. For peptide I, 50 μ l portions of the best pool from

CM-Sephacrose were injected followed by a 30 min linear programme from 30% to 70% solvent B (70% to 30% solvent A) at 1 ml/min flow rate. The variable-wavelength detector was set to 206 nm with a full scale of 1.04 unit, and the fixed detector at 254 nm was set for full scale of 0.024 unit. Fig. 2 shows the initial separation and a re-injection of material recovered from the large peak that emerges at 18.5 min into the programme (54.5% solvent B, 45.5% solvent A). For peptide II, 20–40 μ l portions of the best pool from the CM-Sephacrose chromatography were injected followed by a linear 35 min programme from 10% to 50% solvent B (90% to 50% solvent A) at 1 ml/min. The major peak eluted at 20.5 min into the programme was collected and a portion was re-injected as shown in Fig. 2.

For peptide III, 20 μ l portions of the best pool from the CM-Sephacrose chromatography were injected followed by a linear 20 min programme from 0% to 40% solvent B (100% to 60% solvent A) at 2 ml/min. The major peak eluted at 11 min into

Fig. 2. *H.p.l.c. of samples from ion-exchange chromatography on a Waters C₁₈ μBondapak column in mixtures of water and methanol*

For full experimental details see the text. Panel (a)(i) shows the separation achieved for pepsinogen-(1-12)-peptide; the material represented by the black horizontal bar was collected and a portion was re-injected with the same programme, with the result shown in (a)(ii). Likewise (b)(i) and (b)(ii) show the separation and re-injection respectively of [Gly^{4,5}]pepsinogen-(1-12)-peptide, (c)(i) and (c)(ii) show the separation and re-injection respectively of [Gly^{6,7}]pepsinogen-(1-12)-peptide, and (d)(i) and (d)(ii) show the separation and re-injection of [Gly⁴⁻⁷]pepsinogen-(1-12)-peptide.

Table 1. *Amino acid compositions of synthetic peptides (a) after purification by ion-exchange chromatography and (b) after purification by h.p.l.c.*

For full experimental details see the text. Analyses for serine are uncorrected for decomposition during hydrolysis. The data are the averages for two determinations.

Amino acid	Amino acid composition (mol of residue/mol)			
	Pepsinogen-(1-12)-peptide	[Gly ^{4,5}]pepsinogen-(1-12)-peptide	[Gly ^{6,7}]pepsinogen-(1-12)-peptide	[Gly ⁴⁻⁷]pepsinogen-(1-12)-peptide
(a) After purification by ion-exchange chromatography				
Arg	1.08 [1]	1.10 [1]	1.09 [1]	1.11 [1]
Lys	3.01 [3]	3.32 [3]	2.99 [3]	2.89 [3]
Leu	3.02 [3]	2.94 [3]	2.00 [2]	1.98 [2]
Val	2.97 [3]	1.92 [2]	1.84 [2]	1.04 [1]
Gly	—[0]	2.06 [2]	2.19 [2]	4.13 [4]
Ser	0.65 [1]	0.51 [1]	0.88 [1]	0.85 [1]
Pro	Present [1]	—[0]	Present [1]	—[0]
(b) After purification by h.p.l.c.				
Lys	3.08 [3]	3.01 [3]	3.17 [3]	3.07 [3]
Leu	3.21 [3]	2.97 [3]	1.92 [2]	1.92 [2]
Val	2.79 [3]	2.90 [3]	1.87 [2]	0.96 [1]
Gly	—[0]	1.91 [2]	2.04 [2]	4.36 [4]
Ser	0.79 [1]	0.61 [1]	Present [1]	0.56 [1]
Pro	Present [1]	—[0]	Present [1]	—[0]

the programme (22% solvent B, 78% solvent A) was collected and a portion was re-injected as shown in Fig. 2.

For peptide IV, 15 μ l portions of the best pool from the CM-Sepharose chromatography were injected followed by a linear 20 min programme from 0% to 100% solvent C (100% to 0% solvent A) at 1 ml/min. The major peak eluted at 11 min (38% solvent C, 62% solvent A) had a shoulder on the descending side. Therefore a conservative cut was made from the first quarter of the broad peak. A portion was re-injected as shown in Fig. 2.

Amino acid analyses were performed for the collected peaks from h.p.l.c. and the results are given in Table 1(b).

Inhibition assays

The catalytic activity of free pepsin was determined by using the milk-clotting assay (Dunn *et al.*, 1978). Inhibition assays were performed by mixing a peptide sample in 0.1 M-sodium acetate buffer, pH 5, with a standard amount of pepsin in a constant total volume of 100 μ l, incubating at 37°C for 10 min, then assaying 50 μ l of the mixture for residual pepsin activity. Preliminary studies of the time course of inhibition indicated that equilibrium was reached in 10 min. The resulting activities were always compared with those of standards of pepsin incubated with buffer in the absence of peptide inhibitors under identical conditions and assayed on the same day.

Results and discussion

Synthesis

The synthesis of the peptides by the solid-phase method went exceptionally well. In almost all cases a single coupling was sufficient in each step. In most other syntheses in this laboratory, double couplings have been the rule. The addition of 1-hydroxy-benzotriazole to the coupling reaction is probably responsible for the improvement observed (Konig & Geiger, 1970a,b,c).

The completeness of coupling has been confirmed by the use of fluorescamine assay (Felix & Jimenez, 1973) as well as by the use of a solid-phase sequencing procedure. The sequencing process can yield both qualitative and quantitative information on the mixture of products generated in the coupling reaction (Matsueda *et al.*, 1981). Since peptidyl-resin from the synthesis can be directly introduced into the solid-phase sequencing apparatus, this is a simple and valuable quality-control approach.

Purification

Despite the relative ease of the synthesis, the resulting peptides obtained by HF cleavage of peptidyl-resin are still heterogeneous, as shown by the ion-exchange chromatograms shown in Fig. 1. This

could arise from incomplete deprotection during cleavage. Indeed, we frequently observe (B. M. Dunn & C. Deyrup, unpublished work) that the early fractions in the chromatograms have considerable absorbance at 280 nm; this can only arise from benzyloxycarbonyl or benzyl groups, since the peptides do not possess aromatic amino acids. The amino acid analyses of the middle fractions are also very similar. To choose the best fraction from the ion-exchange procedure, we use amino acid analysis, t.l.c. and inhibition assays. The correct fraction has a composition closest to the predicted, yields a single spot on t.l.c. (with R_f equal to that of the native peptide for the native sequence), and gives the highest ratio of inhibition to concentration.

As a final purification step, we have employed reversed-phase h.p.l.c. with gradient elution. In all four cases we have removed a small amount of impurity by this method.

Fig. 3. Plots of the percentage inhibition observed as a function of the concentration of the synthetic peptide employed

For full experimental details see the text. ○, Pepsinogen-(1-12)-peptide after ion-exchange chromatography; ●, pepsinogen-(1-12)-peptide after h.p.l.c.; ▽, [Gly^{6,7}]pepsinogen-(1-12)-peptide after ion-exchange chromatography; ▼, [Gly^{6,7}]pepsinogen-(1-12)-peptide after h.p.l.c.; □, [Gly^{4,5}]pepsinogen-(1-12)-peptide after ion-exchange chromatography; ■, [Gly^{4,5}]pepsinogen-(1-12)-peptide after h.p.l.c.; △, [Gly⁴⁻⁷]pepsinogen-(1-12)-peptide after ion-exchange chromatography; ▲, [Gly⁴⁻⁷]pepsinogen-(1-12)-peptide after h.p.l.c. The curves in each case are theoretical lines calculated assuming equilibrium binding and using the values of K_1 as the adjustable parameter. The values of K_1 used to generate these four lines are given in Table 2.

Inhibition assays

By measuring the effect of various concentrations of the synthetic peptides on the milk-clotting activity of pepsin, we have determined the apparent inhibition constants for each peptide. The milk-clotting assay is quite reliable and convenient. Use of this method allows us to compare these results with other studies of inhibitors of pepsin also assayed by milk-clotting. The resulting curves are plotted in Fig. 3. In this Figure we have included data from experiments performed with the ion-exchange-purified peptide as well as the h.p.l.c.-purified samples. The activities are virtually identical, which implies that the small amount of impurity present in the ion-exchange-purified peptide samples does not interfere with the interaction with pepsin.

The data in Fig. 3 include all four peptides tested. The data for the better inhibitors are crowded along the ordinate since low concentrations produce high inhibition. The results demonstrate a very large change in inhibitory potency with alteration in the hydrophobic sequence -Val-Pro-Leu-Val-. This is emphasized by plotting the data together in Fig. 3. The lines in Fig. 3 have been calculated by fitting the data for each peptide to a simple equation for equilibrium binding. The values of the apparent equilibrium constant required for the fits are collected in Table 2. Replacing the 4-7 sequence with four glycine residues results in a 350-fold decrease in the affinity of the peptide for pepsin. This should be contrasted with our earlier study of replacements or modification of the basic residues in the pepsinogen-(1-16)-peptide sequence; these resulted in, at most, a 10-fold change in inhibition (Dunn & Deyrup, 1979). The two peptides with two of the four residues replaced have intermediate affinities. These results establish the central role of the 4-7 sequence in the interaction of these inhibitory peptides with pepsin.

In this regard it is noteworthy that, in the pepsinogens from human, Japanese monkey, pig and cow, the hydrophobicity of the 4-7 region is conserved (Kageyama & Takahashi, 1980). As reported by Kageyama & Takahashi (1980), proline-5 and leucine-6 are invariant, though valine-4 is replaced by isoleucine in bovine pepsinogen only,

and valine-7 is replaced by isoleucine in human pepsinogen only.

Finally, it is of importance to stress that the present study has concentrated on analysing the equilibrium effectiveness of the inhibitors. We have recently presented evidence that the first kinetic step of the pepsin-peptide interaction does depend on an ionic interaction (Dunn, 1982). The rapid first step that we have observed (Dunn *et al.*, 1981), by using peptides with fluorescent labels, does not cause inhibition of the enzyme and may be a surface binding event. This is followed by a second, much slower, step that leads to a stable inhibited complex. The assay conditions that we have employed in the present study (incubation at 37°C for 10 min) are sufficient to produce this stable complex for all peptides tested. Therefore the results reported in the present paper deal with differences in the overall equilibrium process. This is dominated by hydrophobic effects, whereas the first step appears to be ionic in nature. This observation of a dependence on hydrophobic binding is consistent with active-site binding by these inhibitors.

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Table 2. Apparent dissociation constants for pepsin-peptide interaction

For details see the text.

Peptide	K_1 (μM)
Pepsinogen-(1-12)-peptide	0.085
[Gly ^{4,5}]pepsinogen-(1-12)-peptide	5.30
[Gly ^{6,7}]pepsinogen-(1-12)-peptide	1.00
[Gly ⁴⁻⁷]pepsinogen-(1-12)-peptide	30.0

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