Improved Pepsin Inhibitor Derived from Activation Peptide 1–16 of Porcine Pepsinogen†

P. M. Harish Kumar and Beatrice Kassell*

ABSTRACT: The peptide Leu-Val-Lys-Val-Pro-Leu-Val-Arg-Lys-Lys-Ser-Leu-Arg-Gln-Asn-Leu, a known pepsin inhibitor, is derived from the first 16 amino acids of porcine pepsinogen. It was prepared from the activation mixture and was modified by guanidination of its three lysine residues to form homoarginine residues. The modified peptide is a better pepsin inhibitor than the native peptide; for 50% inhibition of the milk clotting action of pepsin at pH 5.3, the molar ratio of peptide to pepsin required is 9 for the native inhibitor and only 2 for the guanidinated inhibitor. The dissociation constants (k_I) of the inhibitor–pepsin complexes are 7×10^{-8} and 1.4×10^{-8} M for the native and guanidinated peptides, respectively. The guanidinated peptide is more resistant to digestion by pepsin at pH 3.5. The native and modified peptides partially protect pepsin from inactivation at pH 7. Stepwise removal of the amino-terminal Leu-Val-Har residues from the guanidinated inhibitor by Edman degradation decreases the pepsin-inhibiting activity only slightly at the first step, but markedly at the second and third steps. Thus, all of the amino-terminal sequence except the leucine residue is necessary for full activity.

A pepsin-inhibiting substance formed during the activation of porcine pepsinogen has been known for many years (Herriott, 1938). Based on the finding that in solutions more acid than pH 4, the loss of pepsinogen was not immediately accompanied by an equivalent increase in pepsin, Herriott postulated the scheme shown in Figure 1 for the autocatalytic activation of pepsinogen. A pepsin inhibitor was separated and partially characterized by Herriott (1941) and by Van Vunakis and Herriott (1956, 1957). At that time, the techniques of peptide purification and amino acid analysis had not been perfected, and their analysis does not agree with any part of the amino-terminal sequence later elucidated by Ong and Perlmann (1968). However, Anderson and Harthill (1973) prepared an inhibitor almost corresponding in analysis to the amino-terminal 16 amino acids of porcine pepsinogen. A homologous 17-amino acid peptide from bovine pepsinogen was reported jointly by Foltmann's group and by Kay and Kassell (Harboe et al., 1974), while Kassell et al. (1976) found a peptide from canine pepsinogen that was similar in composition to the first 14 amino acids of bovine pepsinogen. The similarity of the amino-terminal portions of the pepsinogens of the three species, shown in Figure 2, makes it likely that the activation peptides are not simply "throw-aways", but have a physiological role.

The susceptibility of the inhibiting peptides to peptic digestion (Figure 1) renders them unsuitable for investigation of their physiological roles. We have therefore chemically modified peptide 1–16 by guanidination of its lysine residues and have used this derivative to study the basis of the inhibitory activity and one possible physiological role.

Experimental Procedure

Materials. Crystalline pepsinogen was purchased from Sigma. The sulfate of 2-methylisourea was an Eastman product. Phenyl isothiocyanate and trifluoroacetic acid were Sequenal grade, 1-mL vials from Pierce Chemical Co. Solvents for Edman degradation were purified and checked for the absence of aldehydes according to Edman and Begg (1967). Porcine pepsin, 2× crystallized, was obtained from Worthington Biochemicals, Freehold, N.J. Bovine hemoglobin, 2× crystallized, was a Miles product. Pepstatin was a gift from Bristol Laboratories. An immobilized pepsin was prepared according to Goldstein (1973), by coupling an ethylene–maleic anhydride copolymer (Monsanto) with 1,6-diaminohexane and attaching pepsin by means of a water-soluble carbodiimide.

Preparation of Peptide 1–16. The pepsinogen was first purified by chromatography on polylysine-Sepharose 4B at pH 6.5 (Nevaldine and Kassell, 1971). A 600-mg sample of purified pepsinogen was activated at 0 °C and pH 2 for 90 s at a protein concentration of 6 mg per mL. The pH was raised to 3.5, 9.3 mg of pepstatin (equivalent to the dry weight of the pepsinogen) was added, and the activation peptides were separated from the pepsin by the method we previously used for the bovine pepsinogen activation peptides (Kay, 1972; Harboe et al., 1974), i.e. a polylysine-Sepharose 4B column equilibrated with ammonium formate buffer, pH 3.5. The mixture of peptides not retained by the column was lyophilized. The peptides were separated by chromatography on CM-Sephadex C-25 (Pharmacia) at pH 6.0 by a method similar to that of Anderson and Harthill (1973), except that ammonium acetate buffer was used in place of sodium acetate. Pepstatin was recovered in the first peak and peptide 1–16 in the fourth peak (Harish Kumar et al., 1977).

Guanidination of Peptide 1–16. A small scale modification of the method of Chervenka and Wilcox (1956) was used (cf. Kassell and Chow (1966)). The free base form of 2-methylisourea, prepared by precipitating the sulfate with barium hydroxide, was added in 100-fold molar excess to 3 μmol of peptide 1–16 at 5 °C and pH 10.3 in a volume of 1 mL. After 72 h at 5 °C, reagents were removed on a column of Sephadex G-15 (1 × 97 cm, equilibrated with 10 mM ammonium bicarbonate). The peptide peak was lyophilized.

Amino Acid Analysis. Peptides and proteins were hydrolyzed in sealed evacuated tubes with redistilled constant boiling

† From the Department of Biochemistry, The Medical College of Wisconsin, Milwaukee, Wisconsin 53233. *Received March 28, 1977.* Supported by United States Public Health Service Research Grant AM-09826 from the National Institute of Arthritis, Metabolism and Digestive Diseases and by Grant PCM-74-22102 from the National Science Foundation. Presented in part at the First International Conference on Acid Proteases, Structure-Function and Biology, Oklahoma City, Oklahoma, November 21–24, 1976.

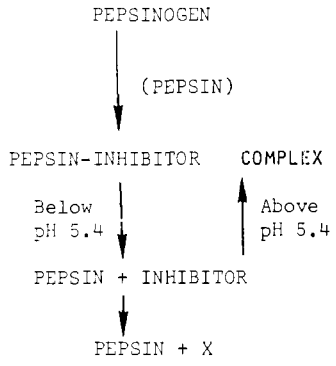

FIGURE 1: The scheme proposed by Herriott (1938) to explain the delay in the formation of active pepsin upon activation of pepsinogen. When the inhibitor dissociates from pepsin, it is gradually digested in the acid solution.

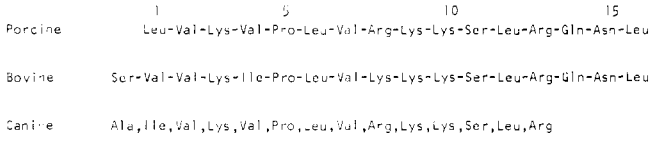

FIGURE 2: Homology in the structure of the amino-terminal portions of porcine, bovine, and canine pepsinogens.

HCl at 110 °C for 24 h or 48 h, or with 4 M methanesulfonic acid containing 0.2% tryptamine (Pierce) at 115 °C for 24 h (Simpson et al., 1976). Analyses were carried out on a modified Beckman 120B amino acid analyzer (Eick et al., 1974).

Milk Clotting Assay of Pepsin-Inhibiting Activity. The activity of the pepsin used was calculated on the basis of 81% of active pepsin by comparison of its hemoglobin-splitting activity with our purest pepsin preparation (Nevaldine and Kassell, 1971).

To determine inhibition of the milk clotting activity of pepsin by peptide 1-16, the turbidimetric method of McPhie (1976) was used with minor modification. We found that a more consistent standard curve for pepsin activity was obtained when the fresh skim milk purchased locally was first centrifuged at 12 000*g* for 10 min in the cold; 2 mL of the centrifuged milk was used instead of 1 mL, diluted as described by McPhie with 44 mL of 0.2 M sodium acetate buffer, pH 5.3, and 4 mL of 0.1 M $CaCl_2$. The diluted milk was prepared each day and stored in the refrigerator, and a small amount was equilibrated at 25 °C before each assay.

We confirmed the observation of Dunn et al. (1976) that peptide 1-16 reacts with pepsin much more rapidly and completely at 37 °C than at 25 °C. However, the turbidimetric assay for pepsin was less consistent at 37 °C than at the 25 °C temperature recommended by McPhie. We, therefore, first incubated the peptide with about 300 ng of pepsin in 1 mL of the pH 5.3 buffer for 15 min at 37 °C and then for 5 min at 25 °C before adding 2 mL of the diluted milk solution at 25 °C to start the assay. There was no increase in inhibition with longer incubations. The standard curve for 50-300 ng of pepsin was prepared in exactly the same way and was rechecked with each new carton of skim milk.

Edman Degradation. A sample of about 1 μmol was degraded essentially by the method of Pederson et al. (1972), but with the entire procedure carried out inside the nitrogen chamber described by Meagher (1975). At each step, following ether extraction of the amino acid thiazolinones (which were discarded), the residual peptide was dissolved in a few tenths of a milliliter of water. Samples were taken for subtractive amino acid analysis (Konigsberg and Hill, 1962) and for determination of pepsin inhibiting activity. The solution was evaporated to dryness under N_2 for the next step.

TABLE I: Amino Acid Composition of Peptide 1-16 and Its Guanidinated Derivative.

Amino acid	Residues per mol		
	Native	Guanidinated	Expected[a]
Lys[b]	3.42	0.13	3
Har		3.25	
Arg	2.00	2.00	2
Asp	1.19	1.18	1
Ser	0.90	1.02	1
Glu	1.14	1.10	1
Pro	1.27	1.20	1
Val	3.07	3.00	3
Leu[b]	4.26	4.17	4

[a] From the sequence of the amino-terminal portion of porcine pepsinogen (Ong and Perlmann, 1968). [b] Slightly high values are due to about 10% contamination with other activation peptides.

Protection of Pepsin from Inactivation at pH 7.0. The enzyme-inhibitor complex was first formed by incubating 0.3 nmol (10 μg) of porcine pepsin with either 3.3 nmol of native peptide or 1.6 nmol of guanidinated peptide in 0.25 mL of 0.2 M sodium acetate buffer, pH 5.3, for 15 min at 37 °C. The pH was adjusted to 7.0, where pepsin alone is rapidly denatured, and the solution was incubated for 10 min before adjustment to pH 2 for hemoglobin assay (Anson, 1939; Chow and Kassell, 1968) of remaining peptic activity. Controls were treated in the same manner, except that buffer of pH 5.3 was used instead of the peptides.

Resistance of the Peptides to Digestion by Pepsin. Immobilized pepsin corresponding in activity to 18 μg of active pepsin was washed with 2 mL of 0.2 M sodium acetate buffer, pH 3.5, and added either to 650 pmol of native peptide or to 160 pmol of the guanidinated peptide in a total volume in 1 mL of the same buffer. The suspension was gently stirred at 37 °C. At various time intervals from 0 to 4 h, the suspension was centrifuged in the cold for 3 min and a 100-μL sample of the supernatant solution was adjusted to pH 5.3 for the milk clotting assay of remaining inhibitory activity.

Results

Table I gives the amino acid composition of the peptide used for these studies. The composition is in agreement with the known sequence of peptide 1-16 (Ong and Perlmann, 1968). In addition to the amino acids listed, there were small amounts of histidine, glycine, and alanine. From the quantities of these amino acids and the known sequence of the total activation fragments (Ong and Perlmann, 1968; Pedersen and Foltmann, 1973; Stepanov et al., 1973), it was ascertained that the product was about 90% pure, the slightly high values for several amino acids being caused by contamination with at least three other peptides. No further purification was considered to be necessary.

Analysis of the guanidinated inhibitor (Table I) indicated more than 96% conversion of the lysine residues to homoarginine, with no change in other amino acids.

Inhibition studies with the native inhibitor and its guanidinated derivative (Figure 3) demonstrated that the guanidinated inhibitor is a stronger pepsin inhibitor. For 50% inhibition, the molar ratio of peptide to pepsin was decreased by the modification from 9 to about 2 (Figure 3a). In Figures 3b and 3c, inhibition of pepsin by the native and modified peptides is plotted by the method of Munck (1976). The K_I values are 7×10^{-8} for the native inhibitor and 1.4×10^{-8} M for the

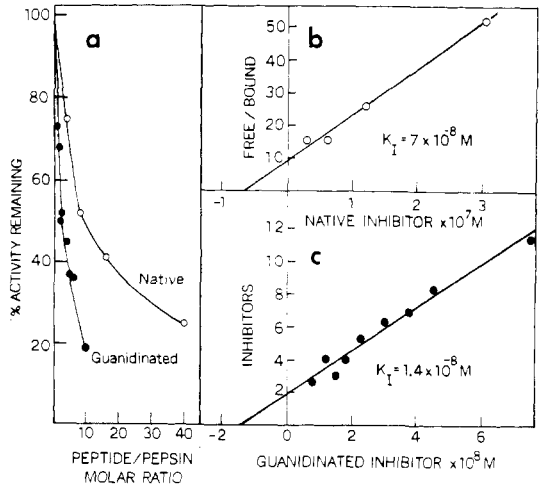

FIGURE 3: Inhibition of 300 ng of pepsin by varying amounts of native peptide 1-16 (O—O) and its guanidinated derivative (●—●). (a) Direct plot; (b and c) plotted according to Munck (1976). The straight lines are calculated by the method of least squares.

TABLE II: Edman Degradation[a] of Guanidinated Peptide 1-16 (Sequence: Leu-Val-Har-Val-).

Edman step	Ratio: Arg = 2		
	Leu	Val	Har
0	4.17	3.00	3.25
1	**3.24**	3.01	3.18
2		**2.05**	3.44
3			**2.55**[b]
4		**1.42**	

[a] The control, carried through the procedure without phenyl isothiocyanate, showed no change in amino acid composition. [b] Incomplete extraction and reconversion during hydrolysis of homoarginine thiazolinone.

guanidinated inhibitor, in reasonable agreement with the K_I of 2×10^{-7} M for the native inhibitor reported by Dunn et al. (1976).

The guanidinated inhibitor retained only one free amino group, which was on the terminal leucine. The absence of other reactive groups made it possible to subject this derivative to the Edman degradation. This permitted evaluation of the role of the amino acids near the amino terminus in the inhibiting activity. Four steps of Edman degradation were carried out as described in Experimental Procedure. Another portion of the material was used as a control of nonspecific loss of activity due to exposure to the Edman reagents; this sample was subjected to identical treatment except for omission of the phenyl isothiocyanate.

Table II shows the sequential removal of the first four amino acids from the guanidinated inhibitor. At step 3, the loss of the homoarginine residue did not appear to be complete. It is likely, however, that cleavage of this residue went further than the value of 2.55 mol indicates, since the thiazolinone of arginine is known to resist extraction and homoarginine thiazolinone would be expected to behave similarly. Acid hydrolysis partially reconverts the thiazolinones or thiohydantoins to the free amino acids (Fraenkel-Conrat et al., 1955), accounting for the high value for homoarginine. Cleavage of the second valine in the fourth step proceeded further than the apparent cleavage of the homoarginine, confirming this explanation. In the control for the Edman reaction, there was no significant change in amino acid composition.

TABLE III: Edman Degradation of Guanidinated Peptide 1-16. Molar Ratio of Peptide to Pepsin for 50% Inhibition.

Step	Amino acid removed	Ratio	Control ratio[a]
0	—	2	2
1	Leu	8	6
2	Val	80	17
3	Har	120	5

[a] The peptide was subjected to the Edman procedure without phenyl isothiocyanate.

TABLE IV: The Effect of Peptide 1-16 and Its Guanidinated Derivative in Protecting Pepsin from Inactivation at pH 7.0.[a]

Molar Ratio peptide: pepsin	Activity remaining (%)	
	Native peptide	Guanidinated peptide
0	0	0
1	16	24
2	29	30
5	30	42

[a] For each ratio of inhibitor to pepsin, the solution was incubated for 15 min at pH 5.3 and 37 °C for formation of the enzyme-inhibitor complex. The pH was adjusted to 7.0 and incubation was continued for 10 min before lowering the pH for assay with hemoglobin substrate.

The results of the pepsin inhibition assays of the degraded guanidinated inhibitor and its control are presented in Table III. After the first step, both the control and the peptide with leucine removed were reduced in activity to about the same degree. Removal of the second and third residues markedly decreased the activity, with no further effect of the cycling on the control peptide. By the third step, some of the remaining activity may possibly be attributed to incomplete removal of the homoarginine residue. The lower inhibitory activity of the degraded peptides was not due to their slower interaction with pepsin. The fourth step was not tested.

Table IV shows that pepsin is partially protected from inactivation at pH 7.0 by the inhibitory peptides. The control solution of pepsin alone lost all activity in 10 min; considerable activity was retained in the presence of both peptide inhibitors, the guanidinated inhibitor being somewhat more effective.

Digestion of the native and guanidinated peptides by immobilized pepsin (see Experimental Procedure) was compared under the optimal digestion conditions determined by Herriott (1938) for loss of inhibiting activity. The activity of the native peptide gradually fell to 71% of its value at zero time. During the same time interval, there was no change in the amount of inhibition of pepsin by the guanidinated peptide. Thus guanidination made the peptide resistant to peptic digestion.

Discussion

Modification of peptide 1-16 of porcine pepsinogen by conversion of its three lysine residues to homoarginine increases its pepsin-inhibiting activity. The availability of an active guanidinated peptide made it possible to determine the role of the residues at the amino terminus. By stepwise Edman degradation, not possible with the native inhibitor containing other free amino groups, it was determined that the terminal leucine is not needed, but the following residues are required for full activity. Dunn and his colleagues (1976) have prepared peptide

1-13 as well as 1-16; both are active inhibitors. Thus, the activity resides between residues 2 to 13.

Gastric juice secretion begins in anticipation of food, so that the empty stomach at the beginning of a meal contains newly activated pepsin. When food of neutral pH mixed with alkaline saliva is swallowed, the pH of the gastric contents rises temporarily until there is further secretion of gastric juice. A physiological role of the activation peptides[1] may be to prevent inactivation of pepsin during the short period of high pH, as suggested by the results in Table IV.

Another role of the peptides may be hormonal in nature. For example, the activation peptide of bovine trypsinogen, Val-Asp_4-Lys, inhibits the secretion of gastric juice (Abita et al., 1973). Thus, when food enters the intestine and gastric secretion is no longer needed, activation of trypsinogen provides an inhibitory peptide.

Herriott (1941) showed that the pepsin inhibitors of the activation peptides are digested by pepsin with a pH maximum near 3.5. We have also found loss of activity of native peptide 1-16 on digestion with pepsin at pH 3.5, but the guanidinated inhibitor is stable under these conditions.

In order to study further the physiological role of the pepsin activation peptides, related peptides that are stronger inhibitors than the native peptides and peptide inhibitors able to resist digestion by pepsin at acid pH are needed. The guanidinated peptide is an approach to solving this problem.

Acknowledgments

We thank Dr. John Kay for the gift of a sample of pepsinogen and Dr. William F. Minor of Bristol Laboratories for a gift of pepstatin.

References

Abita, J.-P., Moulin, A., Lazdunski, M., Hage, G., Palasciano, G., Brasca, A., and Tiscornia, O. (1973), *FEBS Lett. 34,* 251.

Anderson, W., and Harthill, J. E. (1973), *Nature (London) 243,* 417.

Anson, M. L. (1939), *J. Gen. Physiol. 22,* 79.

Chervenka, C. H., and Wilcox, P. E. (1956), *J. Biol. Chem. 222,* 635.

Chow, R. B., and Kassell, B. (1968), *J. Biol. Chem. 243,* 1718.

Dunn, B. M., Moesching, W. G., Trach, M. L., Nolan, R. J., and Gilberts, W. A. (1976), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 35,* 1463.

Edman, P., and Begg, G. (1967), *Eur. J. Biochem. 1,* 80.

Eick, H. E., Ward, P. H., and Kassell, B. (1974), *Anal. Biochem. 59,* 482.

Fraenkel-Conrat, H., Harris, J. I., and Levy, A. L. (1955), *Methods Biochem. Anal. 2,* 359.

Goldstein, L. (1973), *Biochim. Biophys. Acta 327,* 132.

Harboe, M., Andersen, P. M., Foltmann, B., Kay, J., and Kassell, B. (1974), *J. Biol. Chem. 249,* 4487.

Harish Kumar, P. M., Ward, P. H., and Kassell, B. (1977), in Proceedings of the First International Conference on Acid Proteases, Structure-Function and Biology, J. Tang, Ed., New York, N.Y., Plenum Press (in press).

Herriott, R. M. (1938), *J. Gen. Physiol. 22,* 65.

Herriott, R. M. (1941), *J. Gen. Physiol. 24,* 325.

Kassell, B., and Chow, R. B. (1966), *Biochemistry 5,* 3449.

Kassell, B., Wright, C. L., and Ward, P. H. (1976), in Protides of the Biological Fluids, 23rd Colloquium, Peeters, H., Ed., Oxford, Pergamon Press, p 541.

Kay, J. (1972), *Fed. Eur. Biochem. Soc. Meet., [Proc.] 28,* Abstr. 458.

Konigsberg, W., and Hill, R. J. (1962), *J. Biol. Chem. 237,* 2547.

McPhie, P. (1976), *Anal. Biochem. 73,* 258.

Meagher, R. B. (1975), *Anal. Biochem. 67,* 404.

Munck, A. (1976), in Receptors and Mechanism of Action of Steroid Hormones, Part I, Pasqualini, J. R., Ed., New York, N.Y., Marcel Dekker, p 1.

Nevaldine, B., and Kassell, B. (1971), *Biochim. Biophys. Acta 250,* 207.

Ong, E. B., and Perlmann, G. E. (1968), *J. Biol. Chem. 243,* 6104.

Pederson, J. D., Nehrlich, S., Oyer, P. E., and Steiner, D. F. (1972), *J. Biol. Chem. 247,* 4866.

Pederson, V. B., and Foltmann, B. (1973), *FEBS Lett. 35,* 255.

Simpson, R. J., Neuberger, M. R., and Liu, T. Y. (1976), *J. Biol. Chem. 251,* 1936.

Stepanov, V. M., Baratova, L. A., Pugacheva, I. B., Belyanova, L. P., Revina, L. P., and Timokhina, E. A. (1973), *Biochem. Biophys. Res. Commun. 54,* 1164.

Van Vunakis, H., and Herriott, R. M. (1956), *Biochim. Biophys. Acta 22,* 537.

Van Vunakis, H., and Herriott, R. M. (1957), *Biochim. Biophys. Acta 23,* 600.

[1] We are grateful to Dr. I. Michael Samloff of the University of California in Los Angeles for suggesting this function of the inhibiting peptides.