PEPTIDES ISOLATED FROM COLLAGENASE-COLLAGEN DIGESTS

John Manahan and Ines Mandl

Columbia University, College of Physicians and Surgeons
New York

Received March 15, 1961

Digestion of native cattle Achilles tendon collagen with purified Cl. histolyticum collagenase (Mandl et al. 1958) free of unspecific proteinase or peptidase activity yields a number of peptides of various chain length. Separation of these peptides has been attempted in our laboratory by different physicochemical procedures. It soon developed that no one method by itself could completely resolve the complex mixture and that electrophoretically or chromatographically homogenous bands in one system were not necessarily single peptides in other systems or by end group analysis. Combinations of several methods, each dependent on a different principle of separation, gave better results. For the experiments reported here preliminary separation of the collagen digest into five fractions and a supernatant filtrate was effected by methanol coacervation. A 2% aqueous solution of the lyophilized digest cooled to approximately $+7^{\circ}$ was added to an equal volume of absolute methanol at -30° C. As soon as a colloidal suspension formed this was drawn off and further small portions of cold methanol added until no further coacervation could be observed. Each coacervate was filtered, dissolved in a small amount of water and dinitrophenylated by the method of Schroeder et al. (1954). The peptides described here were recovered from only one of these fractions, fraction IV, comprising approximately 10% of the original collagen digest. This preliminary step facilitated the subsequent fractionation but the sequences isolated account for less than 1% of the total collagen and at least some, such as the tripeptide sequence GlyProHypro which is present to a much greater extent in

the collagen molecule (Schrohenloher et al, 1959), undoubtedly recur in other fractions. The 4 peptides listed in Table I are reported at this time in spite of low yields and incomplete analyses because each of them illustrates important points which must be taken into consideration when interpreting studies of collagenase specificity and of the microstructure of the collagen molecule.

The ethyl acetate extract of the dinitrophenylated coacervate was redissolved in methanol and adsorbed on a neutral, Woelm, activity grade I, alumina column. Five bands of DNP-peptides were eluted with solvents of increasing polarity (toluene, methanol, 1% 1.5M pH 6.5 phosphate buffer in methanol, 2% aqu. pyridine, 4% aqu. pyridine, 1.5M phosphate buffer pH 6.5). The peptides in two of these bands, II and V, were further separated by paper electrophoresis in pyridine:acetic acid:water (80:20:900), pH 5.6, at a current density of 0.43 mA/cm and a 400 V potential. The strongest spot in each case was the one migrating farthest towards the positive pole. Eluates from these spots were concentrated and subjected to chromatography on several sheets of 3MM Whatman paper in sec. butanol-3%NH_3 (3:1) in the first dimension, 1.5M phosphate buffer, pH 6.5, in the second dimension. Spots were eluted with methanol or 1% acetic acid and homogeneity of the DNP-peptides tested by electrophoresis in pyridine-acetic acid buffers at three different pH values (3.6, 5.6, 6.5) and rechromatography in several systems. The three most prominent spots out of 9 discernible on chromatograms from alumina column band V are listed as A, C and D in Table I. They account, respectively, for 0.27, 0.05 and 0.03% of total collagen nitrogen or 5, 0.75 and 0.5% of the methanol fraction investigated. The fourth peptide listed (B) was the most prominent on chromatograms from alumina column band II. It accounts for 0.02% of collagen nitrogen, 0.35% of the methanol coacervate fraction. N- and C-terminal amino acids and amino acid composition where known are listed in Table I.

The tripeptide A was isolated in relatively largest amount. This peptide had previously been isolated by Schrohenloher, Ogle and Logan (1959) from collagen digests prepared with a mixture of Cl. histolyticum enzymes. Its presence

TABLE I

AMINO ACID COMPOSITION OF PEPTIDES ISOLATED

	N-terminal	C-terminal	Empirical formula
A.	Glycine	Hydroxyproline	Gly-Pro-Hypro
B.	Glycine	Glycine	?
C.	Alanine	Alanine	$Ala(Gly_8Ala_2Glu_2Asp_2Phe_2Pro_2Thr)Ala$
D.	Glycine	Phenylalanine	$Gly(Gly_6Ala_3Val_2Leu_2Lys_2Glu_2Pro_2AspPheSer)Phe$

in a digest made with purified collagenase free of unspecific proteinase or peptidase proves that it is a primary product of collagenolysis and not formed by secondary degradation. In agreement with others (Heyns and Legler 1959, Kazakova et al. 1958) we found many of the C-terminal amino acids in total collagenolytic digests to be hydroxyproline. It has been known for some time (Schroeder et al. 1954, Kroner et al. 1955) that GlyPro and ProHypro occur in collagen in preference to ProGly and HyproPro sequences. Collagenase of postulated specificity (Nagai and Noda 1959, Heyns and Legler 1959, Nagai et al. 1960) which splits collagen in such a way that bonds one amino acid residue removed from proline are cleaved, would thus be expected to produce the peptide GlyProHypro.

Peptide B was not isolated in sufficient amount for complete amino acid analysis; only its N- and C-terminal amino acids could be established. The C-terminal glycine is unexpected if ProGly sequences are absent in collagen while on the other hand collagenase cleavage is limited to bonds one residue removed from proline. Michaels et al. (1958) who found all other possible amino acids present C-terminal in total collagenolytic digests, did not find any glycine in that position. Our data do, however, indicate that at least some glycine residues are present C-terminal in the total digests. Specificity studies with synthetic substrates showed hydrolysis of CbzHyproGlyGlyProOMe but not CbzProGlyGlyPro (Grassmann et al. 1959). Since the intact collagen is

more similar to the blocked substrate and specificity requirements allow substitution of hydroxyproline for proline, C-terminal glycine is not incompatible with other findings.

The C-terminal amino acid of peptide C is alanine, one of the amino acids found most frequently at the C-terminal end in collagenolytic digests. The N-terminal alanine, however, contrasts with the N-terminal glycine of most other peptides isolated, including the others reported here. On the basis of reports by Michaels et al. (1958) who found only glycine N-terminal in total collagenolytic digests, Grassmann et al. (1959) postulated that solely substrates with the sequence XProRGlyPro were susceptible to collagenase. Nagai et al. (1960 and personal communication) and Heyns and Legler (1960) found that the substrates CbzGlyProLeuAlaPro resp. CbzProAlaAlaProNH$_2$, both containing alanine in place of glycine, were also susceptible to collagenase, though the rate of hydrolysis was much slower than that of the corresponding glycine peptides. Like other workers (Michaels et al. 1958, Kazakova et al. 1958, Heyns and Legler 1959) we have found mostly glycine at the N-terminal position of peptides present in total collagenolytic digests but we also found alanine and other amino acids and feel that the more general formula XPR$_1$R$_2$P better expresses collagenase specificity.

Neither peptide C nor peptide D contain any hydroxyproline; both peptides contain only two proline per chain and according to the postulated specificity requirements (Heyns and Legler 1959, Nagai and Noda 1959) these should be the penultimate residues. Approximately one-third of all residues are glycine, but for quantitative distribution as every third amino acid there is excess of one glycine in peptide C and lack of one glycine in peptide D. The absence of hydroxyproline and the small number of proline residues in peptide sequences 21 and 24 amino acids long are further proof of the heterogeneity of the collagen molecule previously reported from our laboratory and by Grassmann et al. (1956).

This work was supported by grant A779 from the United States Public Health Service.

REFERENCES

Grassmann, W., Hannig, K., Endres, H. and Riedel, A., Z. physiol. Chem., 306, 123 (1956).

Grassmann, W., Hoermann, H., Nordwig, A. and Wuensch, E., Z. physiol. Chem., 316, 287 (1959).

Heyns, K. and Legler, G., Z. physiol. Chem., 315, 288 (1959).

Heyns, K. and Legler, G., Z. physiol. Chem., 321, 184 (1960).

Kazakova, O. V., Orekhovich, V. N. and Shpikiter, V. O., Doklady Akad. Nauk, 122, 657 (1958).

Kroner, T. D., Tabroff, W., and McGarr, J. J., J. Am. Chem. Soc., 77, 3356, (1955).

Mandl, I., Zipper, H. and Ferguson, L. T., Arch. Biochem. Biophys., 74, 465 (1958).

Michaels, S., Gallop, P. M., Seifter, S. and Meilman, E., Biochim. Biophys. Acta, 29, 450 (1958).

Nagai, Y. and Noda, H., Biochim. Biophys. Acta, 34, 288 (1959).

Nagai, Y., Sakakibara, S., Noda, H. and Akabori, S., Biochim. Biophys. Acta, 37, 567 (1960).

Schroeder, W. A., Kay, L. M., Le Gette, J., Honnen, L. and Green, F. C., J. Am. Chem. Soc., 76, 3556 (1954).

Schrohenloher, R. E., Ogle, J. D. and Logan, M. A., J. Biol. Chem., 234, 58 (1959).