The specificity of collagenase

Collagenase is known as a proteinase with a very high degree of substrate specificity; in order to elucidate the nature of this specificity, various synthetic peptides have been investigated as substrates.

MANDL *et al.*[1] tested Leu·Pro·Gly, Pro·Leu·Gly·NH_2, Leu·Phe·Pro, Leu·Phe·Pro·OCH_3, polyproline and several proline- or hydroxyproline-containing dipeptides; GALLOP *et al.*[2] tested polyproline and some origopeptides; MONIER *et al.*[3] and BIDWELL AND VAN HEYNINGEN[4] tried a large number of synthetic substrates with a crude collagenase. None of these compounds were hydrolysed. It has also been established that among proteins only collagen and gelatin are attacked; casein, hemoglobin, serum albumin, fibrin, keratin and elastin do not react.

We have also investigated several synthetic peptides of the type G-P-R[5,6] as possible substrates for purified collagenase[7], namely Pro·Leu·Gly[8], Gly·Pro·Leu[8], poly (Pro·Leu·Gly) with average molecular weight[8] of 3000, and Gly·Pro·Leu·Gly·Pro·NH_2[9]. It was found that the purified collagenase was able to hydrolyze the latter two synthetic peptides. The method of synthesis of these peptides, which are models for collagen, has been published[8]. Their characteristics will be published elsewhere[9].

After hydrolysis of poly (Pro·Leu·Gly) with collagenase, three peptides were found on paper chromatography, with the amino acid sequence Pro·Leu·Gly·Pro·Leu, Gly·Pro·Leu and Gly·Pro·Leu·Gly respectively. The molar ratio for these peptides was 0.8:19:1. No free amino acid could be found among the products of digestion. It is evident that the first peptide was derived from the N-terminal portion of poly-(Pro·Leu·Gly), and the third from the C-terminal portion, and that the polymer was almost completely split into tripeptides. The molar ratio of Gly·Pro·Leu to the tetrapeptide derived from the C-terminal portion was larger than the calculated ratio for the molecular weight of 3000 of the polymer used. This discrepancy may be due to poor recovery of peptides from the ends, or due to an error in the determination of the average molecular weight, or both. There was no evidence of the appearance of Pro·Leu.

Since no glycine and Pro·Leu were found among the products of digestion, and the pentapeptide from the N-terminal portion and the tetrapeptide from the C-terminal portion were found in comparable amounts, it appears that collagenase does not attack Pro·Leu·Gly·Pro·Leu, in which the imino group of N-terminal proline is unmasked. It can be further concluded that the presence of an imino acid only in a position penultimate to glycine does not satisfy the specificity requirements of collagenase, and that two proline residues in the sequence Pro·R.R'·Pro are required.

Gly·Pro·Leu·Gly·Pro·NH_2, which from the above consideration should be the simplest substrate for collagenase, was incubated with purified collagenase. By paper electrophoresis and paper chromatography, it was found that this peptide was split into two peptides with amino acid sequences Gly·Pro·Leu and Gly·Pro·NH_2, respectively. No free amino acid was found.

The above results are consistent with the conclusion that the sequence Pro·R.R'·Pro is necessary in a substrate for collagenase. Other possibilities are, however, not yet completely excluded, and the study of the nature of collagenase specificity is now in progress. Results on the collagenase digestion of purified rat-tail-tendon collagen[7], are in agreement with the present results.

The present authors are greatly indebted to Professor AKABORI and Dr. SAKAKIBARA of Osaka University for their helpful discussions and their kindness in providing us with the necessary peptides.

YUTAKA NAGAI
HARUHIKO NODA

Department of Biophysics and Biochemistry, Faculty of Science, University of Tokyo (Japan)

1 I. MANDL, H. ZIPPER AND L. T. FERGUSON, *Arch. Biochem. Biophys.*, 74 (1958) 465.
2 S. MICHAELS, P. M. GALLOP, S. SEIFTER AND E. MEILMAN, *Biochim. Biophys. Acta*, 29 (1958) 450.
3 R. MONIER, G. LITWACK, M. SOMLO AND B. LABOUESSE, *Biochim. Biophys. Acta*, 18 (1955) 71.
4 E. BIDWELL AND W. E. VAN HEYNINGEN, *Biochem. J.*, 42 (1948) 140.
5 W. A. SCHROEDER, L. M. KAY, J. LEGETTE, L. HONNEN AND F. C. GREEN, *J. Am. Chem. Soc.*, 76 (1954) 3556.
6 M. L. HUGGINS, *Protein Chemistry*, Vol. 5, Kyōritsu Shuppan, Tokyo, 1957, p. 61.
7 Y. NAGAI AND H. NODA, in preparation.
8 H. KITAOKA, S. SAKAKIBARA AND H. TANI, *Bull. Chem. Soc. Japan*, 31 (1958) 802.
9 S. SAKAKIBARA *et al.*, unpublished.

Received March 23rd, 1959

Some chemical changes associated with prothrombin activation

In the activation of purified prothrombin preparations several stages in the development of thrombin activity have been recognized[1]. First, prothrombin loses the capacity to become thrombin in the presence of lung thromboplastin, Ac-globulin and Ca^{++} (2-stage analytical reagents). Then the protein regains its sensitivity to these substances, and lastly thrombin activity arises. The sequence which accounts for experiments completed thus far may be described as follows: Prothrombin (sensitive to Ca^{++} + Ac-globulin + brain thromboplastin) → Prothrombin-derivative I (not sensitive to Ca^{++} + Ac-globulin + brain thromboplastin) → Prothrombin-derivative II (sensitive to Ca^{++} + Ac-globulin + brain thromboplastin) → thrombin and other reaction products.

This unique capacity of the zymogen to undergo modifications implies an intermediate(s) in the activation process. For instance intermediates have been found in the autocatalytic activation of prothrombin in 25 % sodium citrate[2]. We have now found that the first step in prothrombin activation is associated with the appearance of N-terminal proline, and this is true with the use of three different materials; namely, thrombin, purified platelet factor 3, and sedimentable lung thromboplastin.

In the activation with sedimentable lung extract thromboplastin (1 mg/ml) and Ca^{++} (0.023 *M*) about 50 % of the prothrombin (2000 units/ml) was converted to thrombin in 5 h, and the remaining prothrombin became a derivative (not convertable to thrombin in 2-stage analytical reagents). We then removed the thromboplastin by high-speed centrifugation and precipitated the protein by adding acetone to a concentration of 50 % at 0°. The precipitated protein was dried from the frozen state and was found to contain N-terminal proline and glutamic acid. From other experiments it is known that the N-terminal amino acid of bovine prothrombin is alanine[3] and for thrombin it is glutamic acid[1]. A non-thrombin derivative of prothrombin thus has proline as the N-terminal amino acid.