THE SYNTHESIS AND IMMUNOLOGICAL ACTIVITY OF A PEPTIDE RELATED TO COLLAGEN

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The immunological studies with collagen and gelatin have been recently summarized by Seifter and Gallop (1966). Of these, several deal with the characterization of the antigenic determinants of these proteins (Sela and Arnon, 1960; Seifter and Gallop, 1963; Schmitt et al., 1964; Jasin and Glynn, 1965; Steffen and Timpl, 1965; Davison et al., 1967).

In our search for antigenic determinants of collagen an octapeptide having the amino acid sequence Gly-Pro-Gly-Pro-Pro-Gly-Ala-Lys has been synthesized. The peptide exhibited specific precipitin reactions with rabbit antiserum to guinea pig skin collagen. These precipitins could be inhibited by an immunologically active tryptic digest of collagen.

MATERIALS AND METHODS

The peptide was synthesized by the solid phase peptide synthesis method (Merrifield, 1964). The purity of the α-amino tertiary butyloxycarbonyl derivatives of the L-amino acids which were used was ascertained by thin layer chromatography. The ε-amino group of lysine was protected as the carbobenzoxy derivative. Following

cleavage from the resin chromatography of the crude peptide on 1 x 150 cm Dowex 1 x 2 column developed with a buffer system consisting of pyridine-collidine-acetic acid-water ranging from pH 8.8 to 8.1 yielded two peaks. Each peak was electrophoresed at pH 8.8 using pyridine-collidine-acetic acid buffer (20:20:0.13 ml in 1 liter of water) at 30 v/cm. The peptides were located on the electrophoretogram by ninhydrin. The eluted material from the ion exchange column which corresponded to the peptide with the expected electrophoretic mobility was pooled. Analysis of a hydrolysate (6 N HCl at 110°C for 24 hours) on the Beckman Model-120B amino acid analyzer yielded the following amino acid ratios: lysine - 1.05; proline - 2.84; glycine - 2.95; alanine - 1.16. These ratios are substantially in agreement with the theoretical molar ratios for the octapeptide Gly-Pro-Gly-Pro-Pro-Gly-Ala-Lys.

Guinea pig skin collagen was extracted and purified according to the method of Piez et al. (1963). Amino acid analysis of the preparation agreed with those reported for other mammalian collagens (Piez et al., 1963). Twenty mg collagen in 20 ml 0.01 M tris buffer (pH 7.8) was centrifuged at 60,000 g for 60 minutes. The supernatant had a final concentration of 0.3 mg collagen/ml.

One hundred mg collagen in buffer (pH 8.1) consisting of 0.05 M tris and 0.01 M calcium chloride was incubated at room temperature with 2 mg trypsin for 18 hours. The digest was desalted by passage through Sephadex G-10 column, equilibrated and eluted with a solution composed of 0.15 N ammonium acetate in 5% acetic acid. Any undigested material was removed by passage through Sephadex G-75 equilibrated and eluted with the same buffer. Following lyophilization the digest was dissolved in water at a concentration equivalent to 4 mg/ml of collagen from which the digest was derived.

Anti-collagen serum was obtained by multi-site intramuscular

and subcutaneous injections of rabbits with 10 mg collagen in 5 ml 0.05% acetic acid emulsified with an equal volume of Freund's complete adjuvant. The injections were given on alternate weeks for a period of at least 6 months. The animals were bled at least 10 days following an injection. Antisera were dialyzed overnight in the cold against a 10 millimolar solution of tris buffer, pH 7.8.

Qualitative microprecipitin reactions between collagen or peptides and antisera were performed in capillary tubes (1.1 mm inside diameter and 75 mm long), using 20 µl of antigen and an equal volume of antiserum. Inhibition of the precipitin formation by peptides was performed by mixing the antibodies with an equal volume of tryptic digest of collagen, or with the synthetic peptide, prior to the introduction of antigen into the system. The synthetic C-terminal penta- and decapeptides of the tobacco mosaic virus protein (TMVP) tryptic peptide 8 (nomenclature according to Tsugita et al., 1960) were used as controls. These were synthesized in our laboratory as previously described (Young et al., 1967).

RESULTS AND DISCUSSION

Results of the preciptin reactions are summarized in Table I. These show the formation of preciptins between collagen and antiserum obtained from a single rabbit, and the inhibition of this reaction by the tryptic digest of collagen. The specificity of the inhibition by the digest was ascertained in our laboratory by the fact that it could not inhibit the precipitin reaction between bovine serum albumin and homologous rabbit antiserum.[1]

1) The immunological activity of tryptic peptides of calf skin gelatin has been reported by Steffen and Timpl (1965). The immunological activity of a tryptic digest of guinea pig skin collagen has been ascertained in our laboratory (Michaeli, to be published).

Table I

The Immunological Activity of Peptides

Serum	Inhibitor	Antigen	Results
Anti Collagen	-	Collagen	+
Anti Collagen	-	-	-
Control Serum	-	Collagen	-
Anti Collagen	Octapeptide	Collagen	+
Control Serum	Octapeptide	-	-
-	Octapeptide	Collagen	-
Anti Collagen	-	Octapeptide	+
Control Serum	-	Octapeptide	-
Anti Collagen	Tryptic digest	Collagen	-
Anti Collagen	Tryptic digest	Octapeptide	-
Anti Collagen	TMVP pentapeptide	Octapeptide	+
Anti Collagen	TMVP decapeptide	Octapeptide	+

Collagen was used at 160 μg/ml; tryptic digest was used at a concentration equivalent to 4 mg/ml of collagen from which it has been derived; octapeptide, and TMVP penta-and decapeptides were used at a concentration of 220 mμ moles ml.

Qualitative microprecipitin reactions indicated that the precipitin formation between collagen and anti-collagen could not be inhibited even with large excesses of the synthetic peptide (Table I). Data in Table I show that the incubation of the synthetic peptide with anti-collagen resulted in the formation of precipitins and that these precipitins formed only with anti-collagen serum indicating the immunological specificity of this phenomenon. Additional evidence for the immunological specificity of the synthetic peptide is afforded by the finding that the precipitin formation between this peptide and anti-collagen could be completely inhibited by the tryptic digest of collagen but not by synthetic peptides of the tobacco mosaic virus protein. Although the data in Table I were obtained using antiserum from one rabbit, similar results were obtained with antiserum from another rabbit.

The formation of immune precipitins between collagen or gelatin and anti-collagen has been recently ascertained in our laboratory (Michaeli, to be published). However, the formation of precipitin

between an octapeptide and antiserum is unexpected. This phenomenon could result from the aggregation of the peptide or from its adsorption onto serum proteins thus yielding a multivalent antigen. These possibilities are under investigation. The specific binding of the peptide with anti-collagen was very recently confirmed in our laboratory by experiments on the direct binding of the $\left[{}^{14}_{}\mathrm{C}\right]$ internally labeled peptide with anti-collagen sera obtained from two rabbits.

During the preparation of this manuscript it has been brought to our attention that a polymer $(\underline{L}\text{-Pro-Gly-}\underline{L}\text{-Pro})_n$ exhibited immunological activity related to collagen (Sela et al., in press).

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REFERENCES

Davison, P.F., Levine, L., Drake, M.P., Rubin, A., and Bump, S., J. Exptl. Med., 126, 331 (1967).
Jasin, H.E., and Glynn, L.E., Immunology, 8, 95 (1965).
Merrifield, R.B., Biochemistry, 3, 1385 (1964).
Piez, K.A., Eigner, E.A., and Lewis, M.S., Biochemistry, 2, 58 (1963).
Schmitt, F.O., Levine, L., Drake, M.P., Rubin, A.L., Pfahl, D., and Davison, P.F., Proc. Natl. Acad. Sci. U.S., 51, 493 (1964).
Sela, M., and Arnon, R., Biochem. J., 75, 91 (1960).
Sela, M., Schechter, B., Schechter, I., and Borek, F., Cold Spring Harbor Sym., 32, (in press).
Seifter, S., and Gallop, P.M., Collagen Currents, 4, No. 5, p. 40 (1963).
Seifter, S., and Gallop, P.M., In: The proteins (H. Neurath, Ed.), 2nd ed., 4, 153 (1966).
Steffen, C., and Timpl, R.Z., Immunitats-Allergieforsch., 129, 469 (1965).
Tsugita, A., Gish, D.T., Young, J.D., Fraenkel-Conrat, H., Knight, C.A., and Stanley, W.M., Proc. Natl. Acad. Sci. U.S., 46, 1463 (1960).
Young, J.D., Benjamini, E., Stewart, J.M., and Leung, C.Y., Biochemistry, 6, 1455 (1967).