IMMUNOENZYMOTHERY OF CLOSTRIDIUM HISTOLYTICUM COLLAGENASE

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Summary

Rabbits inoculated with purified Clostridium histolyticum collagenase (Clostridiopeptidase A (E.C. 3.44.19) formed precipitating and enzyme activity inhibiting antibodies.

The inhibiting effect of immune γ-globulin and of the immune Fab fragment depends on the antibody to enzyme ratio, on the time of preincubation between these two components, and on the presence of Ca$^{2+}$ during preincubation.

Ca$^{2+}$ appears to be necessary to the formation of the collagenase-anticollagenase antibody complex and implicitly to the inhibition of collagenase activity by the antibody.

Neither the high molecular substrate collagen, nor the small molecular substrate CBZ-hexapeptide protect the collagenase activity against the inhibiting effect of the anticollagenase antibody.

The present paper deals with some immunological properties of Clostridium histolyticum collagenase (Clostridiopeptidase A (EC. 3.44.19), few indications of which were found in literature (1, 2).

A purification procedure and some chemical properties of this enzyme were reported in two previous papers (3, 4).

Materials and Methods

Collagen was supplied by NBCo Laboratories; carbobenzoxy-glycyl-L-prolyl-glycyl-glycyl-L-prolyl-L-alanine$^1$ and the tripeptide glycyl-L-prolyl-L-alanyl were supplied by Mann Research Laboratories; Bio Gel P-300 and P-60 by Bio-Rad Laboratories; Sephadex G-200 by Pharmacia Uppsala; and bovine serum albumin (twice crystallized) by NBCo Laboratories. All other chemicals were analytical-grade products.

Methods

Protein was determined by the method of LOWRY, as modified by MILLER (5), with bovine serum albumin used in plotting the standard curve.

Collagenase assay

Enzyme activity was determined by using the high-molecular undegraded collagen as a substrate, as well as the small molecular CBZ-hexapeptide substrate. With collagen as a substrate the method of Leach (6) was applied, and with CBZ-hexapeptide as a substrate the method of GRASSMAN and NORDWIG (7). Both methods were adapted to our experimental conditions (3,4). When collagen was used as a substrate a calcium-containing buffer, i.e. 0.1 M Na-Veronal-10$^{-3}$ M Ca-acetate, pH 7.2, was added to the system to start collagenase activity. After an incubation of 1 hr at 37° the mixture was inactivated with ethanol. The precipitate formed was discarded by centrifugation. Hydroxyproline (HyP) was determined with the

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$^1$ Abbreviation used: Carbobenzoxy-glycyl-L-prolyl-glycyl-glycyl-L-prolyl-L-alanine = CBZ-hexapeptide.
4-dimethylaminobenzaldehyde reagent on an aliquot of the supernatant.

When CBZ-hexapeptide was used as a substrate 0.1 M Na-citrate 0.1 M Ca-acetate buffer pH 6.3 was used to start enzyme activity. After an incubation of 10 min. at 37° the mixture was inactivated with a 0.1 M trichloracetic acid solution and the released tripeptide glycyl-L-prolyl-L-alanine was determined with a ninhydrine reagent. Both methods were used to determine final residual enzymic activity in each of the experimental systems studied.

Enzyme preparation
Collagenase was isolate from Clostridium histolyticum and purified by ammonium sulphate precipitation, adsorption to and desorption from insoluble collagen and by gel filtration on a Bio-Gel P-300-P-60 column. Details have been given in a previous paper (3).

Preparation of antiserum
Randomly bred California rabbits (weight between 2.0 and 2.5 kg) pre-bled to obtain normal homologous sera were given six intraperitoneal injections of 5 mg alum-precipitated collagenase at weekly intervals. A total of 30 mg enzyme was administered. One week after the last injection the animals were bled out. Sera from each animal were stored in the frozen state until used, and tested separately.

Preparation of immune and normal γ-globulin
γ-globulin from either immune or normal sera was prepared by the method of Perper et al.8.

Preparation of Fab fragments
Fab univalent fragments from normal and immune γ-globulin were obtained by the method of Charlwood and Utsumi9.

Immunochemical methods
Immune γ-globulin was evaluated by double immuno-diffusion in agar gels, by immuno-electrophoresis and by qualitative and quantitative immunoprecipitation reactions according to Kabat and Mayer.12

The quantitative precipitin reaction was carried out in two experimental conditions:

a) Increasing amounts of antigen and constant amounts of antibody, and b) constant amounts of antigen and increasing amounts of antibody. Details of the experimental conditions are given in the legends of the figures.

Precipitates formed after incubation for 1 h at 37° and for 24 hrs at 4° were collected by centrifugation in a refrigerated centrifuge. The tubes corresponding to each dilution were drained and supernatants were kept for determination of residual enzyme activity and for checking antibody or antigen excess. The precipitates were washed twice with an ice-chilled 0.15 M NaCl solution. The amount of precipitated protein was determined after solubilization of the precipitate in 1 ml of 0.1 N NaOH. Control systems containing normal γ-globulin instead of immune γ-globulin were permanently used.

The excess of antibody or of antigen in the supernatants was checked by the "ring" test. Aliquots of each supernatant were distributed into tubes. Antibody was added to one series of tubes and antigen to another. After being kept for 1 h at 37° and overnight at 4°, the tubes displaying precipitin rings indicated an excess of antigen or of antibody while the pairs of tubes without precipitates indicated the point of approximate equivalence.

In other aliquots of the supernatants residual enzyme activity was checked and estimated quantitatively by comparison with the activity of control systems.

Effect of collagenase antibodies on the enzymic activity of collagenase
To test this effect experiments were conducted in the following experimental conditions: a) Preincubation for 1 hr and then for 18 hrs at 4° of a constant amount of antibody (1 mg immune γ-globulin) with various amounts of antigen (10 μg to 300 μg of enzyme) so as to include the zone of antibody excess, the equivalence point, and the zone of antigen excess. After incubation the precipitates formed and the corresponding supernatants were separated by centrifugation. Enzyme residual activity was checked in the washed precipitates as well as in the supernatants after addition of the substrate and of a suitable buffer. Control systems containing normal γ-globulin instead of immune γ-globulin were included. b) A constant amount of antigen (300 μg enzyme) and various amounts of antibody (0.5 mg to 2.5 mg

1 Collection of the Dr. I. Cantacuzino Institute, Bucharest, Romania.