STUDIES ON THE SPECIFICITY OF BACTERIAL COLLAGENASE*

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Summary

The specificity of Clostridium histolyticum Collagenases A and B has been investigated employing a cyanogen bromide peptide of known sequence obtained from chick skin collagen. The enzyme A cleaves only between the X and Gly bond in the sequence-Pro-X-Gly-Pro-Y-.

The collagenase from Clostridium histolyticum has been studied by a number of laboratories (1, 2). It was originally suggested that this enzyme attacked only collagen (3) and peptides both natural and synthetic with the sequence-Pro-X-Gly-Pro-Y-. The X can be any amino acid but most frequently is Ala or Hypro (4, 5, 6, 7, 8,) and Y may be any amino acid residue. Recently papers have appeared which indicate that the enzyme specificity is not as strict as was previously proposed (9, 10). These conflicting reports concerning the specificity prompted us to examine the possibility that the specificity of the two forms of the bacterial collagenase,

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A and B, might be different. Our data reported here indicate that enzyme A hydrolyzes only the X-Gly bond in the Pro-X-Gly-Pro-Y- sequence and that enzyme B is capable of hydrolyzing many other bonds, and suggest that the previously reported differences in the specificity may be due to the enzyme form used in those investigations.

Materials and Methods

Collagenase A and Collagenase B were prepared as previously described (11, 12).

Clostridium histolyticum collagenase Worthington CLSPA grade was chromatographed on a DEAE cellulose column (DE 52, Whatman) 1.5 x 24 cm at room temperature. Fractions of 1 ml were collected every 2 minutes and absorbance of the effluent was measured at 280 mμ with an LKB ultraviolet absorptiometer. Collagenase A was eluted as the first protein peak with 0.06 M KPO_4 buffer, pH 7.4 and Collagenase B was obtained as a second peak of protein with 0.6 M acetate buffer, pH 5.6. The ascending and descending portions of each protein peak were pooled separately resulting in four fractions. These fractions were dialyzed versus 0.01 M Tris, 0.001 M $CaCl_2$, pH 7.4 at 4°. The ascending portion of Collagenase A was employed in these studies. This fraction gave a single protein band on polyacrylamide disc electrophoresis by the method of Davis and Ornstein (13)(14). One precipitin line was obtained by immunodiffusion of the enzyme versus antibody directed toward Collagenase A.

The cyanogen bromide peptide of chick skin collagen α1-CB2, was prepared by the procedure described previously (15). The criteria of their purity and the amino acid sequence of the peptide were also previously reported (16).

Collagenase A (20 μgm) and cyanogen bromide peptide (2.0 mg) were incubated at 37° in0.05 M Tris, 0.005 M $CaCl_2$, pH 7.4 in 1.0 ml final volume. Aliquots (100 μl) were taken at one hour intervals, placed in 25% acetic acid (50 μl) to terminate the reaction and kept in an ice bath. The reaction products were examined by descending paper chromatography using Whatman #1 paper and n-butanol-acetic acid-water (4:1:5) with Gly-Pro-Ala as a marker. After development the peptides were visualized by staining with 0.2% ninhydrin in acetone. The products were also fractionated on a Bio-Gel P-4 column (2.2 x 40 cm) equilibrated with 0.1 N acetic acid and the effluent was monitored at 230 mμ. The amino acid compositions of the separated products were determined after hydrolysis in constant boiling HCl at 108° for 24 hours in a nitrogen atmosphere (17).

Results and Discussion

In Figure 1a is presented the Bio-Gel P-4 chromatogram of a Collagenase A digest of α1-CB2. Two well separated peaks were observed. The first peak, Col A2, consisted of a peptide fragment with amino acid composition consistent with the COOH-terminal 33 residues of α1-CB2 whereas the second peak, Col A1, contained a tripeptide of one residue each of Gly, Pro and Ala. This observation was further substantiated by paper chromatography of the reaction product with authentic tripeptide Gly-Pro-Ala. Even on prolonged incubation (24 hours) no other products could be detected.

In contrast, Collagenase B under the same reaction conditions catalyzed a more extensive hydrolysis. A Bio-Gel P-4 chromatogram of a collagenase B digest of α1-CB2 is depicted in Figure 1b. The material in the first peak was identified as the dodecapeptide, α1-CB2-Col B7

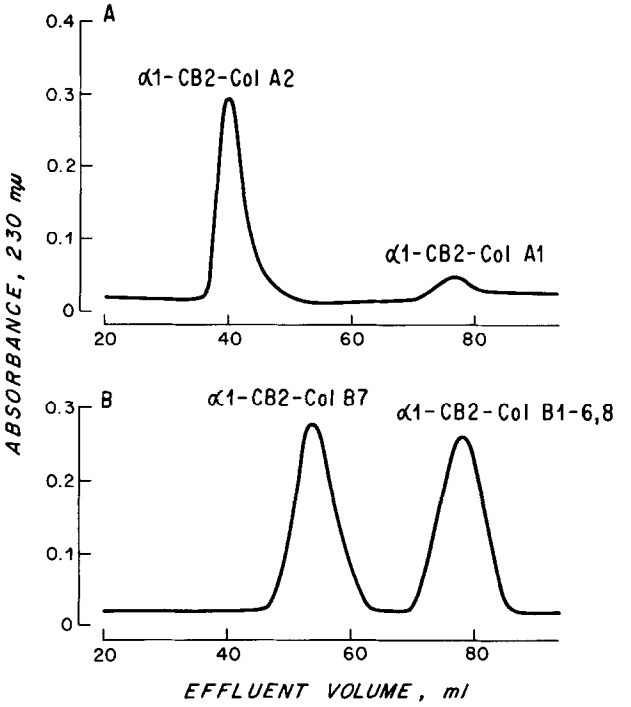

Figure 1a. Separation of peptides resulting from collagenase A digestion of chick skin α1-CB2 on a Bio-Gel P-4 Column. α1-CB2-Col A2 contains 33 amino acid residues. α1-CB2-Col A1 is the tripeptide Gly-Pro-Ala.

Figure 1b. Separation of Collagenase B digest of chick skin α1-CB2 on a Bio-Gel P-4 column. α1-CB2-Col B7 contains 12 amino acid residues. The second peak consists of a mixture of 7 small peptides.

(see Figure 2). The second peak contained a mixture of seven small peptides. These peptides were resolved by a combination of cation exchange chromatography on Spherix (Pheonix Instrument) and paper chromatography into seven pure peptides (Col B1, 2, 3, 4, 5, 6 and 8) (see Figure 2). The details of the latter procedure and the resulting chromatogram were previously reported (16) and therefore, not reproduced here. Thus the specificity of the Collagenase B was identical

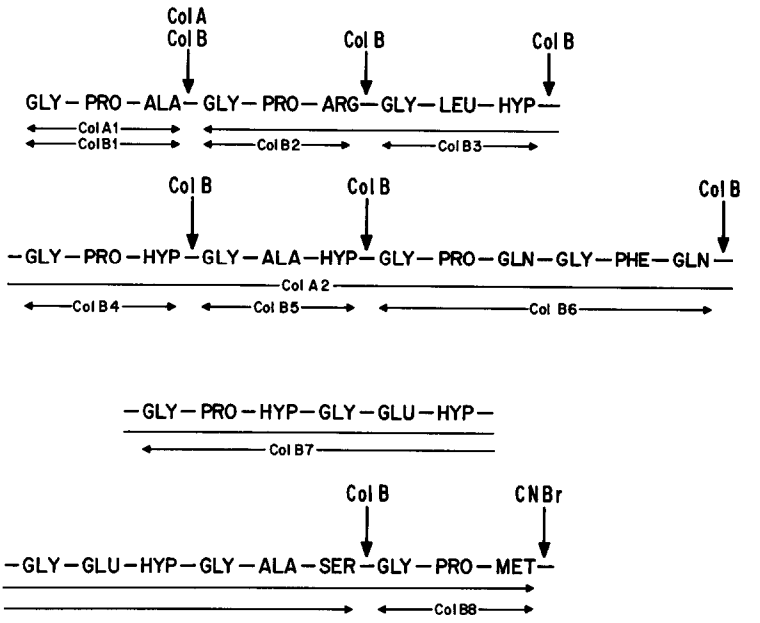

Figure 2. Sequence of α1-CB2 from chick skin collagen (16). Col A stands for the point of cleavage by Collagenase A. Col B, points of scission by collagenase B.

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with that reported by Bornstein (9) on his studies on rat skin α1-CB2 and also with that reported later by Kang and Gross (16). The apparent broader specificity of the Collagenase B was not a reflection of a rate phenomenon as evidenced by our inability to demonstrate the appearance of the tripeptide Gly-Pro-Ala in the absence of other reaction products in aliquots taken after limited incubation periods.

Figure 2 summarizes the sequence of α1-CB2 as determined by Kang and Gross (16) and the points of cleavage by the enzymes A and B.

We conclude that the Collagenase A prepared from Clostridium histolyticum cleaves only the bond between the X and Gly in a sequence-Pro-X-Gly-Pro-Y-, whereas the Collagenase B is far less specific and cleaves in addition between the Y-Gly bond in sequences-Gly-X-Y-Gly-Pro-Z- or

Gly-X-Y-Gly-Z-Hyp-. Thus the reports of an apparent lack of specificity by bacterial collagenase may be due to the fact that the enzyme had not been fractionated into its two components. Furthermore, use of the specific forms of bacterial enzyme should prove helpful in future investigations of the primary structure of collagen.

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