BBA 63394

## Collagenase action on the synthetic tripeptide polymers, $(Gly\text{-}Pro\text{-}Ala)_n$ and $(Gly\text{-}Ala\text{-}Pro)_n$

The forms of collagenase (EC 3.4.4.19) found in culture filtrates of several species of Clostridium are rather specific enzymes for the hydrolysis of selected bonds in either denatured or native collagens of various origins (for reviews, see refs. 1 and 2). The substrate specificity of clostridial collagenase has in the past been generalized[1,2] as hydrolysis of the Y–Gly bond in sequences of the form Z–Pro–Y–Gly–Pro–X where Z is a blocking group or an amino acid covering the amino terminus of proline, where hydroxyproline can replace either or both of the proline residues[3–5], and where X may be a blocking group, any of a number of amino acids, or may be absent[4]. This generalization has been modified as follows: Glycine may be replaced by alanine, as suggested both by the presence of some N-terminal alanine in collagenase digests[6,7] and by the hydrolysis of appropriate synthetic peptides[8,9]. In addition the imino acid in either position shown in the model sequence above may be absent[4,10], although reports of peptide substrates lacking an imino acid in both 'proline' positions shown above have not been encountered by the present authors.

In connection with other studies, we have had recent occasion to examine the products of collagenase action on two synthetic tripeptide polymers, $(Gly\text{–}Pro\text{–}Ala)_n$ and $(Gly\text{–}Ala\text{–}Pro)_n$. A single tripeptide product was identified in each case. $(Gly\text{–}Pro\text{–}Ala)_n$ yielded the tripeptide Gly–Pro–Ala, entirely as would be expected from many earlier studies of collagenase specificity. The product of $(Gly\text{–}Ala\text{–}Pro)_n$ hydrolysis might have been anticipated as the tripeptide Ala–Pro–Gly, since this would satisfy the apparent selectivity of the enzyme for clearing bonds one residue removed from an imino acid and would also conform to the reported susceptibility of bonds involving the amino group of alanine. However, the only product found was the tripeptide Gly–Ala–Pro, indicating both the precedence of N-terminal glycine over second-position proline as a determinant of collagenase specificity, and the freedom from a requirement for proline or hydroxyproline in either of the positions shown in the generalized model.

The polymer $(Gly\text{–}Pro\text{–}Ala)_n$ was a commercial product prepared by Fox Chemical Co. (Los Angeles, Calif.), after the method of ORIEL AND BLOUT[11]. This material was examined in Dr. BLOUT's laboratory, and gave a circular dichroism spectrum in trifluoroethanol identical with that of the 14 000 molecular weight polymer reported[11], but was less water-soluble than the original preparation of ORIEL AND BLOUT (personal communication from Dr. H. FOX). The polymer $(Gly\text{–}Ala\text{–}Pro)_n$ was prepared by Miles–Yeda and was reported to have a molecular weight of 8400. Both polymers were passed through a 2 cm × 37 cm column of polyacrylamide gel (Biogel P-30). $(Gly\text{–}Ala\text{–}Pro)_n$ and $(Gly\text{–}Pro\text{–}Ala)_n$ both emerged as broad but rather symmetrical peaks centering, respectively at 6 ml and 15 ml behind the void volume (40 ml). The tripeptide Gly–Pro–Ala emerged from the same column 120 ml behind the void volume.

The tripeptide, Gly–Pro–Ala, was obtained from the Fox Chemical Co., while Gly–Ala–Pro was generously provided by Dr. A. Marglin of the National Heart Institute. Each tripeptide was pure as judged by homogeneity on thin-layer chro-

matography (*n*-butanol–acetic acid–water, 4:1:5, by vol.), paper electrophoresis (pyridine–acetate buffer (pH 3.5), other conditions as noted earlier[5]), and ion-exchange chromatography (Technicon amino acid analysis system (pH 3.49), Biorad resin A-5, 0.6 cm × 75 cm, 60°, 4.5 h run). Each tripeptide had the expected amino acid composition by automatic amino acid analysis following acid hydrolysis, and each gave the expected sequence analysis by identification of N-terminal amino acids as the dansyl derivatives following successive degradation by the Edman method, using the specific methods noted earlier[5].

Collagenase digestion mixtures for either polymer contained in a final volume of 4 ml: 5 mg of polymer, 0.2 mg of purified collagenase (CLSPA, Worthington), 200 μmoles of Tris (pH 7.4) and 20 μmoles of $CaCl_2$. The mixture was incubated at 30° and small samples, taken at intervals, were acidified by adding 0.5 vol. of 0.1 M HCl. The reaction was followed by ninhydrin assay of such samples by the method of TROLL AND CANNAN[12]. The time course of digestion is shown for each polymer in Fig. 1. Correcting for the small blank ninhydrin value of collagenase and applying

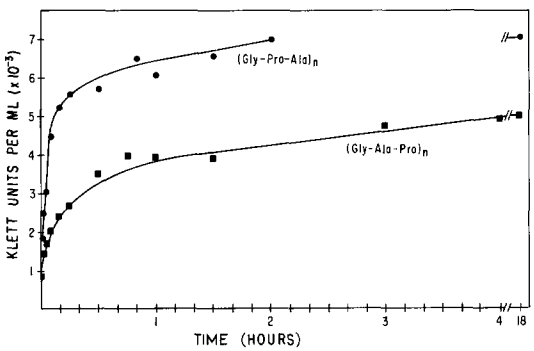


Fig. 1. Time course of digestion by collagenase of the tripeptide polymers $(Gly–Pro–Ala)_n$ and $(Gly–Ala–Pro)_n$. Ordinate units are Klett units (in thousands) per ml of reaction mixture, representing absorbance with the 56 filter in the assay for ninhydrin color. Theoretical Klett units per ml for complete conversion of $(Gly–Pro–Ala)_n$ to Gly–Pro–Ala were 9500; for complete conversion of $(Gly–Ala–Pro)_n$ to Gly–Ala–Pro, theoretical Klett units per ml were 6100. See text for other details of the digestion procedure.

the ninhydrin color yields of the individual tripeptides (about 76% and 53% of the color yield of glutamic acid, for Gly–Pro–Ala and Gly–Ala–Pro, respectively) it was estimated that hydrolysis had proceeded to between 70% and 75% of complete release of each polymer as the constituent free tripeptides containing N-terminal glycine. The digestion product of $(Gly–Pro–Ala)_n$ (both after 2 h and 18 h of digestion) appeared as a single ninhydrin-positive spot on thin-layer chromatography (solvent cited above), cochromatographing with reference Gly–Pro–Ala. It behaved similarly on paper electrophoresis (conditions cited above), and after mixture with reference Gly–Pro–Ala, emerged from the amino acid analyzer column as a single symmetrical peak about 153 min after hydroxyproline in the pH 3.49 buffer system noted above. Sequence analysis agreed with the identification of the product as Gly–Pro–Ala.

On paper electrophoresis at pH 3.5, the digestion product of $(Gly–Ala–Pro)_n$ also gave a single ninhydrin-positive spot which was not resolved from Gly–Pro–Ala. However, the product of $(Gly–Ala–Pro)_n$ digestion differed from Gly–Pro–Ala on ion-exchange chromatography. In the amino acid analyzer with the pH 3.49 buffer system, after mixture with reference Gly–Ala–Pro, it emerged 112 min after hydroxyproline, as a single symmetrical peak. Sequence analysis of the digestion product was consistent with the designation Gly–Ala–Pro.

The identification of Gly–Ala–Pro as the only significant hydrolysis product of the polymer $(Gly–Ala–Pro)_n$ provides new information concerning the specificity of collagenase, demonstrating that an imino acid may be absent from both proline positions indicated in the model sequence, Z–Pro–Y–Gly–Pro–X. Two structural features generally considered to influence the site of collagenase action are in conflict in $(Gly–Ala–Pro)_n$: one is the position of the two prolines, that could direct cleavage of the bond one residue removed from each; the other is the position of glycine which (in conjunction with other structurally permissive features) directs cleavage of the bond involving its own amino terminus. It is clear that in the present case the latter specificity takes complete precedence over the former, even though collagenase is believed capable of cleaving bonds involving the amino group of alanine.

The present findings may be considered to confirm and extend recent results of BORNSTEIN[19], based on a study of a 36-residue peptide of known sequence derived from rat collagen. Thus BORNSTEIN reported collagenase cleavage of bonds to release the tripeptides Gly–Leu–Hyp and Gly–Ala–Hyp, suggesting that a terminal imino residue in a triplet beginning with glycine might be sufficient to permit collagenase action at the bond involving the N-terminus of glycine in that triplet. However, it is important to note that in both these cases the preceding triplet contained a second-position proline so that it could not be decided whether the terminal imino residue was in itself sufficient to permit collagenase action. The release of Gly–Ala–Pro from $(Gly–Ala–Pro)_n$ extends BORNSTEIN's findings by showing that indeed a second-position imino acid in the preceding triplet is not essential. It is not yet clear, however, whether, alternatively, a third position imino residue is required in the preceding triplet, *i.e.*, whether the Y–Gly bond would be cleaved in the sequence Gly–X–Y–Gly–Z–Pro, where neither X, Y, nor Z was an imino residue.

Any consideration of clostridial collagenase specificity is complicated by the knowledge that given preparations may consist of several different forms of the enzyme[13–18], each with possibly varying substrate specificity. Further, some preparations include, presumably as a contaminating enzyme, trypsin-like protease activity[20] probably unrelated to a specificity for collagen. In the present experiments the freedom of the enzyme preparation from significant activity on non-collagenous protein (Catalog, Worthington Biochemical Co.; also see ref. 21) and the rapidity and completeness of the release of Gly–Ala–Pro as the only recognized product, suggest that the hydrolytic specificity demonstrated here might also be expected in the action of collagenase on native or denatured collagen.

The increasing availability of ordered polymers such as those employed here, as well as the use of long sequences of collagen, as in the case studied by BORNSTEIN[19], should permit a more complete assessment of the priorities of various sequences for collagenase specificity.

This work was supported by Grant GM-11105 from the U.S. Public Health Service.

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Received March 12th, 1969

*Biochim. Biophys. Acta*, 185 (1969) 251–254