Glycylhydroxyprolyl Sequences in Earthworm Cuticle Collagen: Glycylhydroxyprolylserine*

AIDA GOLDSTEIN‡ and ELIJAH ADAMS

From the Department of Biological Chemistry, University of Maryland School of Medicine, Baltimore, Maryland 21201

SUMMARY

A second tripeptide representing a glycylhydroxyprolyl sequence, glycylhydroxyprolylserine, has been isolated from purified earthworm cuticle collagen following digestion of the denatured collagen by clostridial collagenase. This tripeptide was estimated to represent 4 to 5% of the total hydroxyproline in the peptide mixture released by collagenase action. The isomeric tripeptide, glycylserlyhydroxyproline, was not detected but could have been present at a maximum concentration representing another 4% of the total peptide hydroxyproline. Data are presented on the susceptibility of native and denatured earthworm cuticle collagen to clostridial collagenase and on the amino acid composition and chromatographic behavior of a number of peptide fractions obtained from collagenase digests of earthworm cuticle collagen.

Evidence from sequence data on vertebrate collagens (1–4) and from both natural and synthetic polymers tested with vertebrate proline hydroxylases (5–7) suggests that only those prolines in position three of repeated tripeptide sequences beginning with NH2-terminal glycine (Gly–X–Pro) can be hydroxylated. Earthworm cuticle collagen, which has the highest reported hydroxyproline to proline ratio, was examined to determine whether the above generalization held for this unusual collagen as well. In a previous communication (8), we reported the occurrence of frequent glycylhydroxyproline sequences in earthworm cuticle collagen. This conclusion was based on sequence data on vertebrate collagens (1–4) and from both natural and synthetic polymers tested with vertebrate proline hydroxylases (5–7) suggests that only those prolines in position three of repeated tripeptide sequences beginning with NH2-terminal glycine (Gly–X–Pro) can be hydroxylated.

The purpose of this paper is to describe in more adequate detail some of the methods and findings briefly summarized earlier (8) and to report the identification of a second tripeptide, glycylhydroxyprolylserine, in collagenase digests of cuticle collagen.

EXPERIMENTAL PROCEDURE

Earthworms were obtained as noted earlier (8). Cuticles were easily removed from the worms after use of a freeze-thaw procedure. Tap water-rinsed worms were placed in plastic bags, suspended in a Dry Ice-ethanol bath until frozen solid, thawed at 5°C, and then suspended in distilled water at room temperature for at least 30 min. The worms were then placed on Parafilm. The cuticles, which were readily peeled off with forceps, were placed in cold distilled water. Cuticles were soaked at least 2 hours at 5°C with frequent water changes to loosen cellular material. Each cuticle was then placed on Parafilm, scraped with a spatula to remove all adhering cellular material, and then re-washed in cold distilled water. At this stage, the cuticles were of a uniform texture and had a white, opalescent appearance.

Purified salt-soluble cuticle collagen was prepared by the method of Jesse and Harrington (9) with the modification noted earlier (8). Purified acid-soluble cuticle collagen was prepared by a modification of the method of Maser and Rice (10). The residue, after initial sodium chloride extraction, was extracted by stirring overnight in 0.5% acetic acid (approximately 1.5 ml per cuticle). The soluble material was adjusted to 5% NaCl by addition of 20% NaCl solution. The precipitated collagen was collected by centrifugation and resuspended by stirring overnight at 5°C in 0.5% acetic acid (three-quarters the volume of the first extraction). The precipitation and re-solution sequence was repeated once, and the final solution, in 0.5% acetic acid at one-third the initial volume, was dialyzed against changes of water (2 liters each) and stored at −15°C.

Crude acid-soluble collagen was prepared by stirring cleaned cuticles overnight at 5°C in 0.5% acetic acid. After centrifugation, the soluble collagen was dialyzed exhaustively against distilled water. Calf skin collagen (acid-soluble collagen, Grade A), purchased from Calbiochem, was dissolved (2 mg per ml) in 0.5% acetic acid by stirring overnight at 5°C. The extract was centrifuged, although solution appeared complete, and the supernatant solution was dialyzed against several changes of water in the cold.

The reference tripeptides, glycylhydroxy-L-prolyl-L-serine and glycyl-L-serlyhydroxy-L-proline, which were purchased from Fox Chemical Corporation (Los Angeles, California), were checked both by amino acid analysis and sequence determination by a combination of Edman degradation and the dansyl proce-
NaCl-soluble collagen and purified acetic acid-soluble collagen were prepared as described (see Reference 8 and "Experimental Procedure"). Crude acid-soluble collagen was obtained directly from cleaned, washed cuticles as described in the text, and without further salt precipitation. All figures are residues per 1000 residues.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Purified NaCl-soluble</th>
<th>Purified acetic acid-soluble collagen</th>
<th>Crude acetic acid-soluble collagen</th>
</tr>
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<tr>
<td></td>
<td>Peptidea</td>
<td>Collagenb</td>
<td>Peptidec</td>
</tr>
<tr>
<td>Hyp</td>
<td>161 ± 15</td>
<td>161 ± 14</td>
<td>150 ± 15</td>
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<tr>
<td>Asp</td>
<td>58 ± 3</td>
<td>60 ± 0</td>
<td>66 ± 7</td>
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<tr>
<td>Thr</td>
<td>49 ± 5</td>
<td>49 ± 4</td>
<td>55 ± 6</td>
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<tr>
<td>Ser</td>
<td>88 ± 5</td>
<td>87 ± 4</td>
<td>93 ± 9</td>
</tr>
<tr>
<td>Gla</td>
<td>81 ± 3</td>
<td>81 ± 2</td>
<td>81 ± 4</td>
</tr>
<tr>
<td>Pro</td>
<td>9 ± 1</td>
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<tr>
<td>Gly</td>
<td>349 ± 13</td>
<td>349 ± 7</td>
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<tr>
<td>Ala</td>
<td>101 ± 13</td>
<td>100 ± 2</td>
<td>95 ± 9</td>
</tr>
<tr>
<td>Val</td>
<td>16 ± 3</td>
<td>18 ± 4</td>
<td>27 ± 3</td>
</tr>
<tr>
<td>Ile</td>
<td>15 ± 1</td>
<td>13 ± 3</td>
<td>17 ± 2</td>
</tr>
<tr>
<td>Leu</td>
<td>28 ± 2</td>
<td>26 ± 1</td>
<td>33 ± 3</td>
</tr>
<tr>
<td>Tyr</td>
<td>1 ± 0</td>
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<tr>
<td>Phe</td>
<td>6 ± 1</td>
<td>5 ± 1</td>
<td>8 ± 1</td>
</tr>
<tr>
<td>Lys</td>
<td>16 ± 1</td>
<td>16 ± 1</td>
<td>17 ± 2</td>
</tr>
<tr>
<td>His</td>
<td>1 ± 1</td>
<td>2 ± 0</td>
<td>3 ± 2</td>
</tr>
<tr>
<td>Arg</td>
<td>23 ± 1</td>
<td>23 ± 2</td>
<td>24 ± 3</td>
</tr>
</tbody>
</table>

a Average values and range for two analyses on two samples.
b Average values and range for five analyses on three samples.
c Released in ethanol-soluble fraction by collagenase; see text.
d Single determination.

were assayed by the ninhydrin method and also were acidified and subjected to amino acid analysis.

RESULTS

Composition of Various Collagen Fractions—Cleaned cuticles used to prepare purified salt- or acid-soluble collagen appear to be essentially free of extraneous tissue as judged by the uniform white, almost translucent, color of the cuticle, and the fact that they were completely soluble in dilute (0.5%) acetic acid. Both purified salt-soluble and purified acid-soluble cuticle collagen preparations have similar amino acid compositions, as shown in Table I. In contrast, cleaned whole cuticles that had been dissolved in 0.5% acetic acid without further purification by salt precipitation showed appreciably lower values for glycine and hydroxyproline and higher values for tyrosine (Table I). The similarity in amino acid analyses for purified collagen initially extracted from the cuticle by either dilute sodium chloride or dilute acetic acid encouraged us to use the latter preparations for much of the work described, particularly since the final yield of the latter type of preparation exceeded (by 5- to 10-fold) that of purified salt-soluble cuticle collagen. The amino acids, 3-hydroxyproline, hydroxylysine, and methionine, which have been reported as absent, or low, and variable in other analyses (9, 10, 13), were not explicitly measured for the data of Table I. More recent findings1 suggest that crude acetic acid-soluble collagen shows relatively high values for apparent methionine (up to 5 residues/1000 residues) and that all three types of preparation contain some 3-hydroxyproline and hydroxylysine.

1 E. Adams and P. Bornstein, unpublished studies.
While this paper was being prepared, a report by Muir and Lee appeared (14) containing a similar comparison of crude large peptides that are soluble in 75% ethanol and that contain natured earthworm cuticle collagen.

Results agree generally with theirs, particularly in the similar amino acid analyses of purified salt- or acid-extracted collagen and in the higher aromatic amino acid content of crude compared with purified preparations. Small differences, especially in the analytical values for crude collagen, may relate to the completeness of removal of adherent epithelium (15, 16).

Digestion with Collagenase—To permit a study of the sequence position of hydroxyproline relative to glycine, it was necessary to degrade the collagen into peptides small enough to be conveniently sequenced. This was accomplished by treating purified earthworm cuticle collagen with clostridial collagenase, which releases predominantly short peptides with NH₂-terminal glycine, as previously reported (8). A striking characteristic of collagenase digestion of earthworm cuticle collagen is the marked contrast between the susceptibility to collagenase action of native versus heat-denatured cuticle collagen, shown in Fig. 1. While heat denaturation also enhances the digestibility by collagenase of calf skin collagen, the effect of denaturation is much less marked than for earthworm cuticle collagen (Fig. 2); the generalization seems valid that, without denaturation, earthworm cuticle collagen would appear to have negligible substrate activity with clostridial collagenase. In the case of denatured earthworm cuticle collagen, the rapid release of hydroxyproline, compared with the slower release of ninhydrin-positive material (Fig. 1), suggests that collagenase acts first to release relatively large peptides that are soluble in 75% ethanol and that contain essentially all of the hydroxyproline; these peptides are then further degraded into smaller peptides. It was possible to increase the rate of hydrolysis of earthworm cuticle collagen by colla-
largely position 2 hydroxyproline. Because the amino acid peptidase. This finding suggested that Peak I-4 contained the following treatment of pooled Peak I (Fig. 3) with leucine aminopeptidase. Peak I-4 appeared relatively uninfluenced in elution position by the gradient used, but it was not identified in pooled samples of Peak K by a paper electrophoretic system known to resolve Gly-Hyp-Ala and Gly-Ala-Hyp (8). The isomeric peptide, Gly-Ala-Hyp, should have been eluted in the same position as the NH₂-terminal residues in the peptide mixture, and that 50% of the hydroxyproline occurs in position 2 from the NH₂-terminus. It is difficult to state with confidence whether the 22% of hydroxyproline estimated to occur in position 3 of the peptide mixture was real or was a carryover from incomplete Edman degradation of position 2.

Isolation of Specific Peptides—The approach to fractionating peptides from collagenase digests, by using the Technicon elution system and collecting approximately three-fourths of the column outflow with a fraction collector, was indicated earlier (8). For convenience, earlier data (Fig. 1 of Reference 8) are reproduced here (Fig. 3); detailed data on the composition of each of the first 11 peaks (A to K) are given in Table II.

Peak K—The identification of Peak K as consisting primarily of Gly-Hyp-Ala was documented earlier (8). The isomeric peptide, Gly-Ala-Hyp, should have been eluted in the same position by the gradient used, but it was not identified in pooled samples of Peak K by a paper electrophoretic system known to resolve Gly-Hyp-Ala and Gly-Ala-Hyp (8).

Peak I—Fig. 3 and Table II indicate that Peak I is complex. Separation of the peptides of Peak I and rechromatography under the conditions noted for Fig. 1 of Reference 8, but with an increase in the volume of buffer in each chamber to 100 ml, produced four partly separated peaks. Of these, only the fourth peak (I-4) appeared relatively uninfluenced in elution position following treatment of pooled Peak I (Fig. 3) with leucine aminopeptidase. This finding suggested that Peak I-4 contained a tripeptide of glycine, hydroxyproline, and serine, with about 20% contamination by other amino acids. The I-4B peak could also be obtained in position 2 from the NH₂ terminus. It is difficult to state with confidence whether the 22% of hydroxyproline estimated to occur in position 3 of the peptide mixture was real or was a carryover from incomplete Edman degradation of position 2.

Table III

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Amount through analyzer</th>
<th>Amount relative to hydroxyproline</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>I-4A</td>
<td>I-4B</td>
</tr>
<tr>
<td>Hyp</td>
<td>0.056</td>
<td>0.015</td>
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<tr>
<td>Asp</td>
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<td>0.023</td>
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<tr>
<td>Thr</td>
<td>0.004</td>
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<tr>
<td>Ser</td>
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<tr>
<td>Gln</td>
<td>0.018</td>
<td>0.003</td>
</tr>
<tr>
<td>Gly</td>
<td>0.050</td>
<td>0.019</td>
</tr>
<tr>
<td>Ala</td>
<td>0.008</td>
<td>0.014</td>
</tr>
</tbody>
</table>

Fig. 5. Thin layer chromatogram of dansyl derivatives of the first two positions of Peak 1-4B. The solvent was chloroform-tert-butyl alcohol-acetic acid (70:30:1). The numbered starting positions represent addition of: 1, position 1 dansyl derivative of reference Gly-Hyp-Ser; 2, position 1 dansyl derivative of I-4B; 3, reference dansylglycine; 4, position 2 dansyl derivative of reference Gly-Hyp-Ser; 5, position 2 dansyl derivative of I-4B; 6, reference dansylhydroxyproline; 7, reference dansylglycine, dansylhydroxyproline, and dansylserine. Spots were made visible by ultraviolet light.
rectly from pooled Peak I by rechromatography with the Aminex resin by the citrate-methanol (pH 2.75) system.

Identification of Gly-Hyp-Ser—The verification of Peak 1-4B as Gly-Hyp-Ser by the dansyl-Edman method is shown in Fig. 5. As a further check on the identity of Peak 1-4B, it was compared with three reference tripeptides purchased from Fox Chemical Corporation (Gly-Hyp-Ser, Gly-Ser-Hyp, and Gly-Hyp-Glu). Column chromatography of the reference tripeptides with the Aminex resin, pH 2.75, citrate-methanol system showed that only the first of these was eluted in the position of Peak 1-4B, while the latter two were eluted later in the region of Peak J (Fig. 3). The isomeric tripeptides Gly-Hyp-Ser and Gly-Ser-Hyp were resolved on thin layer chromatography with a solvent system of ethanol-water (75:25). Both 1-4B and Gly-Hyp-Ser reference peptide migrate identically on thin layer chromatography, and both give a yellow color when sprayed with ninhydrin. Parenthetically, spots of three reference tripeptides containing position 2 hydroxyproline turned yellow when sprayed with ninhydrin, while the isomeric tripeptides with position 3 hydroxyproline were purple, seemingly a convenient and useful distinction.

Frequency of Gly-Hyp-Ser—It was reported earlier that Gly-Hyp-Ala accounts for 9 to 10% of the hydroxyproline in collagenase digests of earthworm cuticle collagen and that little or no Gly Ala Hyp was present as such in the peptide mixtures studied (8). In the case of Gly-Hyp-Ser, the large losses attendant on a multistep isolation procedure made it more difficult to estimate the frequency of this tripeptide in collagenase digests. Peak I represents at least 20% of the peptide hydroxyproline (Table II) and, from other data, Peak 1-4 contains about 80% of the hydroxyproline of Peak I or 16% of the total. For purposes of purity, Peak 1-4B was separated from 1-4A in a manner favoring the latter but detrimental to recovery of Gly-Hyp-Ser. The Gly-Hyp-Ser of Peak 1-4B was estimated to represent 4 to 5% of the total hydroxyproline in the peptide mixture.

The possibility exists that an appreciable quantity of the isomeric tripeptide, Gly-Ser-Hyp, might have been present in the collagen-derived peptides. This tripeptide would be expected in Peak J (Fig. 3); the data of Table II indicate that, if all of the serine and hydroxyproline of that peak were assumed to be Gly-Ser-Hyp, the latter would also represent about 4% of the total hydroxyproline. The possible presence of Gly-Hyp-Ser or Gly-Ser-Hyp as a mono-, di-, or trigalactoside (17) was not investigated. The conclusion that the peptide mainly composing Peak 1-4B is Gly-Hyp-Ser, without substitution by galactose, is based on the identity of its chromatographic and electrophoretic behavior with reference Gly-Hyp-Ser.

DISCUSSION

The data of the previous (8) and the present reports indicate the occurrence of a substantial quantity of so-called position 2 hydroxyproline in both mixed and isolated peptides. This represents the first substantial indication that hydroxylation of proline residues in certain collagens is not confined to prolines occurring in position 3 of the triplet sequences beginning with glycine. The generalization that hydroxyproline occurs only in position 3, on the other hand, is well supported by a variety of data from vertebrate collagen (1-7).

It should be noted that the possibility of hydroxyproline frequently occurring in position 2 of earthworm cuticle collagen was earlier raised (19, 18) by the qualitative evidence for susceptibility of this collagen to clostridial collagenase, coupled with the relatively low proline content and the then current belief that a position 2 imino residue was required for the release by collagenase of NH2-terminal glycine triplets. Parenthetically, this argument might have been countered by the more recent finding that Gly-ALA-Pro, yields much Gly-Ala-Pro by collagenase action (19); hence, the hydroxyproline of earthworm cuticle collagen might still, hypothetically, be confined to position 3 without requiring insusceptibility of this collagen to collagenase.

An independent basis for predicting the frequent occurrence of hydroxyproline in position 2 in earthworm cuticle collagen was a consideration of the apparent requirement for an imino residue in that position, based on the measured thermal stability of this collagen (20). The predicted frequency (about 50%) coincides with the value obtained by sequence analysis of the mixed peptides (8). The quantitative uncertainties of the Edman method applied to a mixture of peptides (3), however, should caution against any serious comparison of theory with the frequency found by this method.

Two reports indicate the possibility that, in vertebrate collagen, position 2 proline may also be hydroxylated, although infrequently. One is the reported isolation from bovine Achilles tendon collagen by Wolf, Logan, and Ogle (21) of Gly-3-Hyp-4-Hyp; the other is the recent report (22) of a small percentage of position 2 hydroxyproline, as indicated by the Edman sequence method applied to peptides released by collagenase action on insoluble Achilles tendon. It should be noted, however, that the limitations of sequencing mixed peptides raise doubts concerning the attribution of a low frequency of any residue to a specific position. The more clear-cut data derived by sequencing specifically defined peptides isolated from vertebrate collagen α chains have to date indicated no exception to the generalization that, in vertebrate collagen, hydroxyproline is confined to position 3 of glycine triplets (4, 23, 24).

The frequent presence of position 2 hydroxyproline in earthworm cuticle collagen but not in vertebrate collagen may reflect, at least in part, a difference in specificity of the respective hydroxylating enzymes. This is supported by unpublished data from this laboratory (studies made with a partly purified hydroxylase from the subcuticular epithelium of earthworms and the ordered polymers (Gly-Pro-Ala)n and (Gly-Ala-Pro)n, and also by recently reported findings of Nordwig and Pfab (25) in experiments with crude earthworm cuticle extracts and the polymer (Pro-Gly-Pro)n which was specifically labeled in either of the two proline positions.

REFERENCES

Glycylhydroxyprolyl Sequences in Earthworm Cuticle Collagen: Glycylhydroxyprolylserine
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