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Characterization of Vertebrate Collagenase Activity by High-Performance Liquid Chromatography Using a Synthetic Substrate

ROBERT D. GRAY AND HOSSAIN H. SANEII

Department of Biochemistry, University of Louisville School of Medicine, Louisville, Kentucky 40292

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The hydrolysis of the model collagenase substrate, 2,4-dinitrophenyl-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg by partially purified tadpole back-skin collagenase was monitored by separation of the substrate peptide from the product peptides 2,4-dinitrophenyl-Pro-Gln-Gly and Ile-Ala-Gly-Gln-D-Arg by reverse-phase high-performance liquid chromatography. The method provides a sensitive, relatively rapid means of determination of collagenase activity using purified enzyme samples. It is not by itself, however, suitable for use with impure systems since the tissue culture medium from tadpole back skin was found to contain at least three peptidases which could be separated by gel filtration and which showed identical high-performance liquid chromatographic elution profiles using the octapeptide model substrate, but only one of which cleaved triple helical collagen.

Tissue collagenases initiate collagen degradation by making a specific cleavage between residues 775 and 776 in triple helical collagen (1). In type I collagen, the susceptible bond is Gly–Ile in the α_1 chain and Gly–Leu in the homologous α_2 chain. Interest in the biochemistry of tissue collagenases is increasing as a result of the recognition of the probable role of these enzymes in several pathological processes, including corneal ulceration, rheumatoid arthritis, periodontal disease, and tumor invasion (2).

Several different assay systems for collagenase activity which utilize the native substrate have been developed. Recently, however, Masui *et al.* (3) reported the synthesis of the model octapeptide, DNP[1]-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg, which has an amino acid sequence identical to that flanking the collagenase-susceptible bond in the α_1(I) chain of collagen. This peptide derivative is cleaved by tadpole back-skin collagenase and by rheumatoid synovial collagenase at the Gly–Ile bond to produce two product peptides: DNP-Pro-Gln-Gly and Ile-Ala-Gly-Gln-D-Arg. The rate of hydrolysis is estimated by partitioning the product tripeptide derivative into ethyl acetate followed by measuring the absorbance at 365 nm (3). This method is obviously nonspecific since if other peptidases are present which produce small DNP-peptides, these might also be extracted into the organic phase with the resultant overestimation of collagenase activity. It would appear that analysis of the reaction mixture by a high-resolution technique such as high-performance liquid chromatography would alleviate such a problem by showing the presence of peptides other than those expected from collagenase activity.

The present paper describes a method which utilizes the superior sensitivity and resolving power afforded by high-performance liquid chromatography (HPLC) to separate and quantitate both the disappearance of the substrate peptide and the appearance of the two product peptides. In addition, we found that a serious limitation

[1] Abbreviations used: bis-tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; DNP, 2,4-dinitrophenyl, HPLC, high-performance liquid chromatography.

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in the use of the model peptide in impure systems is the potential appearance of peptidases which have the same specificity as collagenase toward the model substrate, but lack activity against triple helical collagen.

MATERIALS AND METHODS

Instrumentation. The unhydrolyzed substrate was separated from the two product peptides at ambient temperature using a Varian Associates Model 5020 liquid chromatograph equipped with a 4-mm × 30-cm C_{18} reverse-phase column (Varian MCH-10, 10-μm particle size). Column effluent was continuously monitored during the separation with an LKB Model 2138 uv monitor equipped with an HPLC flow cell of pathlength 2.5 mm and a volume of 8.0 μl. The wavelength was 206 nm. The absorbance was recorded on a strip chart in which full-scale absorbance of 0.05 was displayed on a 200-mm chart running at a rate of 0.33 in./min. The sample was applied to the column through a Valco (Houston, Tex.) Model CV-6-UHPa-N60 manual injector with a 10.0-μl sample loop. The analytical column was preceded by a 2-mm × 4-cm guard column manually packed with Vydac Reverse-Phase Pellicular packing.

Chemicals. Acid-soluble calf skin collagen was from Sigma Chemical Company, St. Louis, Missouri. HPLC-grade water and acetonitrile were obtained from Burdick and Jackson Laboratories (Muskegon, Mich.). Phosphoric acid (85%) was reagent grade from Matheson, Coleman and Bell (Norwood, Ohio). The aqueous phase (0.1% H_3PO_4) was filtered through a 0.45-μm Millipore filter before use. Other chemicals were reagent grade or better.

The vertebrate collagenase substrate, DNP-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg was obtained either from Peninsula Laboratories (San Carlos, Calif.) or from United States Biochemicals (Cleveland, Ohio). The product peptide, DNP-Pro-Gln-Gly was from either Peninsula Laboratories or from the Protein Research Foundation (Osaka, Japan). Stock solutions of these compounds were prepared at approximately 3 and 1 mM for the substrate and product derivatives, respectively, by warming in HPLC-grade distilled water. Aliquots of the stock solutions were frozen at −20°C in containers which were wrapped in aluminum foil to protect the photosensitive DNP-peptides from exposure to the room light. The concentration of the DNP derivatives was determined spectrophotometrically at 372 nm by diluting 0.01 ml of the stock solution into 0.40 ml 1% $NaHCO_3$ and reading the absorbance in 2-mm cuvettes with a Cary 219 spectrophotometer. An absorption coefficient of 16.0 mM^{-1} cm^{-1} was assumed for each compound (4).

Vertebrate collagenase was partially purified from lyophilized tissue culture medium of the back skin removed from *Rana catesbeiana* tadpoles essentially as described by Nagai *et al.* (5). The medium was harvested, the fraction precipitating between 20 and 50% of saturation with ammonium sulfate collected by centrifugation, dissolved in 0.05 M Tris, 0.15 M NaCl, 5.0 mM $CaCl_2$, pH 7.6, and the active fraction recovered after gel filtration on a 2.5 × 40-cm column of Sephadex G-200 using the above Tris buffer as eluant. The specific activity of this preparation was 310 nmol of DNP product formed/min/mg protein. The preparation degraded acid-soluble calf skin collagen producing a typical cleavage pattern consisting of the TC^A and TC^B fragments. The specific activity was approximately 10 μg/min/ml as determined using ^{14}C-acetylated collagen (New England Nuclear) as substrate, separating the reaction products using electrophoresis on 6% sodium dodecyl sulfate-polyacrylamide gels (6), and excising the TC^A bands for scintillation counting. Protein concentration was estimated using the Bradford (7) assay with Coomassie brilliant blue G-250 and bovine gamma globulin as standard (Bio-Rad, Richmond, Calif.).

Collagenase assays were carried out in either 0.05 M Tris-HCl, 0.15 M NaCl, 5 mM

$CaCl_2$, pH 7.6, or in 0.05 M bis-tris-HCl, 0.15 M NaCl, 5 mM $CaCl_2$, pH 6.5. Assays using the DNP-octapeptide substrate were conducted at 37°C and those using calf skin collagen were measured at 25°C.

Procedures. Stock solutions of substrate and buffer were diluted with deionized water into 0.1-ml conical, stoppered polyethylene centrifuge tubes which had been cut back to a length of about 15 mm. The reaction was initiated by adding the enzyme; after mixing, the reaction vessel was tightly stoppered and submerged in a constant-temperature bath. The total volume of the reaction mixture varied between 10 and 25 μl, depending on the number of samples to be removed for injection onto the HPLC column. Successive aliquots of from 3 to 10 μl were removed at timed intervals with a 10.0-μl Valco injection syringe. The sample was not treated further, but was immediately applied to the column which had previously been equilibrated for at least 3 min with a mobile phase consisting of 0.1% H_3PO_4/CH_3CN (80:20, v:v) at a flow rate of 2.0 ml/min (8). The reverse-phase column was washed with the starting solvent for 2 min, after which the proportion of CH_3CN was increased linearly up to 40% by volume over a 12-min time period. This gradient in organic solvent resulted in the elution of both the DNP-tripeptide product and the unreacted DNP-octapeptide substrate. The column was washed with 0.1% H_3PO_4/CH_3CN (60:40, v:v) for an additional 3.0 min, after which the initial solvent conditions were restored for a period of at least 3 min prior to injection of the next sample. The time interval between assays was thus 18 to 20 min.

Quantitation of results. The quantity of each peptide was determined from each reaction mixture by comparison of the observed peak height with a standard curve constructed using the authentic DNP-tripeptide product and the DNP-octapeptide in a solution which contained all the components of the reaction mixture except collagenase. The linear regression program of a Texas Instruments TI-55 calculator was used to determine the best fit of the standards to a straight line.

RESULTS

The separation of authentic DNP-Pro-Gln-Gly from the DNP-octapeptide substrate DNP-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg is illustrated in Fig. 1 by the two peaks (labeled A and B) resolved at 8.3 and 13.9 min, respectively. The peptides were dissolved in Tris-buffered reaction medium; the peaks emerging at 1.0 and 1.4 min were substances present in the medium since they were present when the buffer alone was injected and were absent when aqueous solutions of the individual peptide derivatives were applied to the column. In addition, the small peak emerging in this and in subsequent chromatograms after the DNP-substrate peak appeared when water alone was injected and is therefore probably an impurity in the solvent.

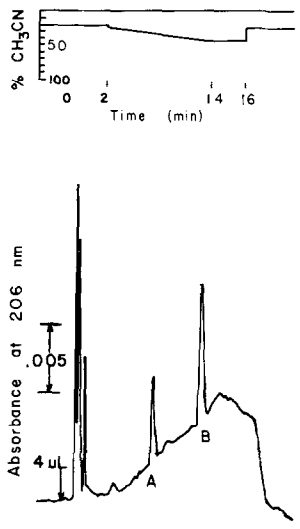

FIG. 1. Separation of DNP-Pro-Gln-Gly (A) from DNP-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg (B) using reverse-phase HPLC. An aliquot of 4.0 μl was injected at the arrow. The initial solvent was 0.1% H_3PO_4/CH_2CN (80:20). The column was eluted with this solvent for 2.0 min, following which the CH_3CN was increased to 40% linearly over a 12-min time period. The flow rate was 2.0 ml/min. The upper graph shows the gradient used.

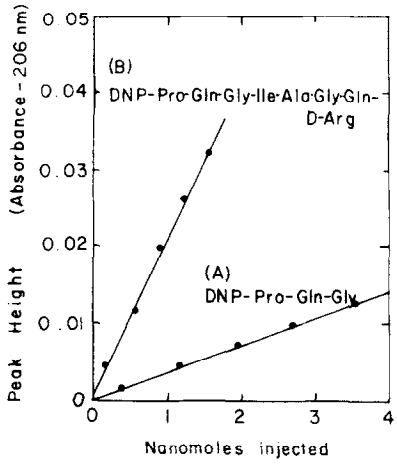

FIG. 2. Standard curve showing peak height vs nanomoles of DNP-product (A) and DNP-substrate peptide injected (B), using the conditions of Fig. 1. Lines determined by linear regression.

The increasing baseline absorption in Fig. 1 results from an increasing percentage of acetonitrile (which has a small absorbance at 206 nm) in the mobile phase. Obviously, a flat base line would be superior from an analytical standpoint. However, we could not find suitable isocratic solvent conditions which would allow adequate resolution of the three expected peptides (DNP substrate, DNP product tripeptide, and the pentapeptide product) in a reasonably short time. Since 206 nm was the only suitable wavelength available on our instrument, we found it necessary to accomodate the presence of an increasing baseline. A change in wavelength to 215 nm would still give good sensitivity but would not be in the range of background absorbance from the organic solvent.

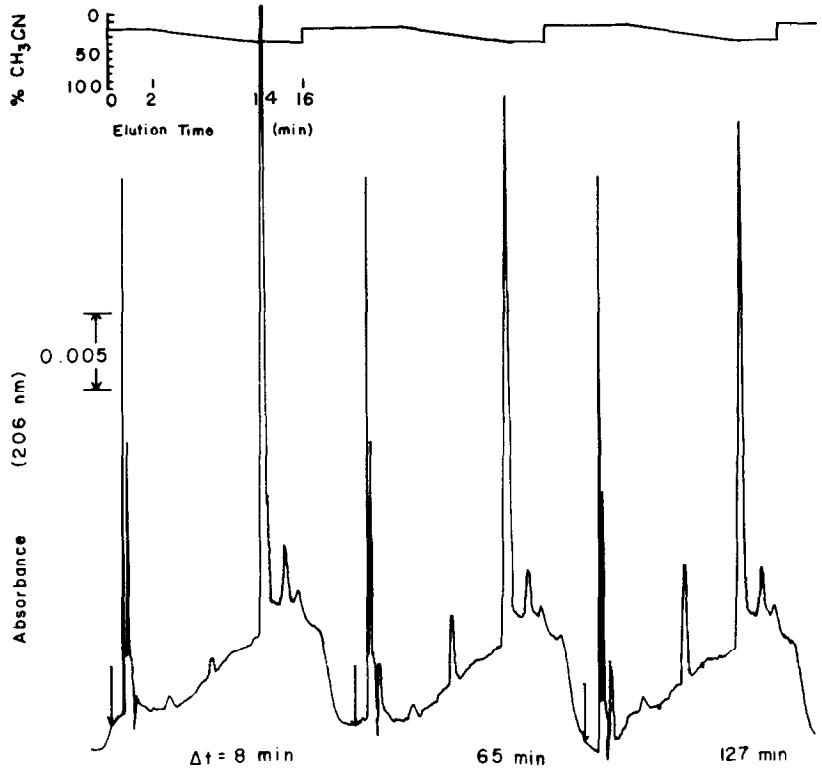

FIG. 3. Separation of hydrolysis products of the action of tadpole collagenase on DNP-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg by reverse-phase HPLC. The upper tracing shows the gradient employed. The reaction was initiated at zero time by the addition of 4 μg of enzyme. At the indicated times, a 4.0-μl aliquot of the reaction mixture was injected on the HPLC column and the peptides separated under the conditions outlined in Fig. 2. Conditions: pH 6.5 assay system.

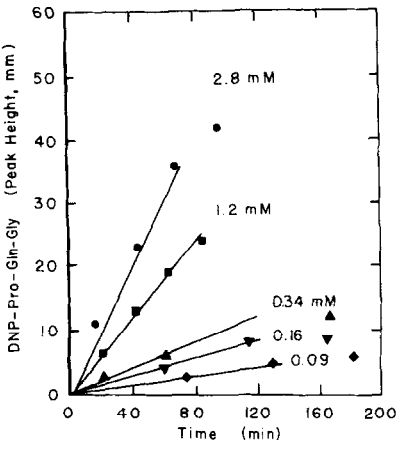

FIG. 4. Dependence of the rate of hydrolysis of DNP-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg on substrate concentration. Conditions: pH 7.6 assay system; 4.2 μg enzyme protein.

Alternatively, monitoring at 365 nm would also have permitted estimation of the DNP derivatives with sensitivity about equal to that achieved at 206 nm. Methanol/0.1% H_3PO_4 was also tried as a solvent system, but the background absorbance of methanol at 206 nm was greater than that of acetonitrile.

A standard curve relating peak height (as measured by the vertical distance from the apparent absorbance maximum on the chart to extrapolated base line) is shown in Fig. 2. The slope of the standard curve for the octapeptide derivative is greater than that for the tripeptide derivative, in keeping with the larger number of chromophoric peptide groups in the larger derivative.

Figure 3 shows elution profiles obtained at timed intervals throughout a typical reaction. The height of peak at 8.3 min, identified by cochromatography with authentic DNP-Pro-Gln-Gly, obviously increases with time, while the height of the substrate peak at 13.9 min decreases. In addition, the peak at 2.5 min also increases with time. This peak is assumed to be the pentapeptide product, Ile-Ala-Gly-Gln-D-Arg. The remaining peaks, whose heights were constant throughout the reaction, are unknown components of the buffer or of the enzyme solution and were identified as such by appropriate controls.

The time dependence of the disappearance of the DNP-octapeptide is shown in Fig. 4 for several different initial concentrations of substrate. At all concentrations tested, the appearance of the product was linear for at least 60 min. A reciprocal plot of this data is given in Fig. 5 from which a K_m value of 1.7 mM and a V of 310 nmol/min/mg protein was estimated for this preparation of enzyme under the conditions of the assay at pH 7.6 and 37°C.

The initial rate of product formation was also a linear function of enzyme concentration. These data are illustrated in Fig. 6, where the data for a 10-fold range of protein concentrations are shown.

Finally, during the course of purification of the crude tissue culture concentrate by gel filtration on Sephadex G-200, we found that three fractions eluted which exhibited high peptidase activity toward the DNP-octapeptide. Only one of these had significant collagenase activity. The data supporting this conclusion are illustrated in Fig. 7 which shows a typical activity–elution profile of the crude 20–50% ammonium sulfate fraction

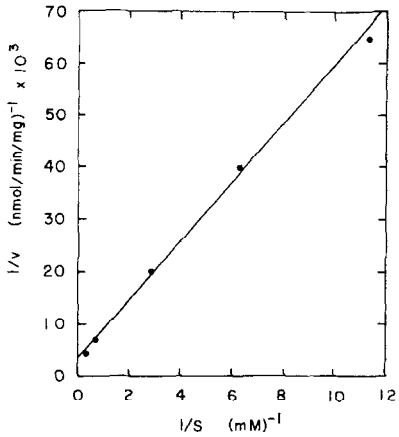

FIG. 5. Lineweaver–Burk plot of data in Fig. 4 for determination of K_m and V of tadpole collagenase at pH 7.6, 37°C, using HPLC assay. The value of K_m is 1.7 mM.

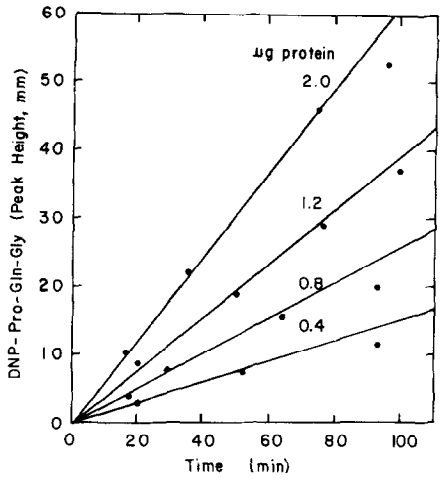

FIG. 6. Dependence of rate of hydrolysis of DNP-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg on collagenase concentration. Substrate concentration was 1.7 mM, pH 7.6, 37°C.

of tadpole back-skin tissue culture medium chromatographed on Sephadex G-200. As demonstrated in Fig. 8, only fraction III, which was eluted at a volume corresponding to approximately 40,000 molecular weight, degraded calf skin collagen to produce the characteristic TC^A and TC^B fragments. The apparent molecular weight of the collagenase-containing peak corresponds to that expected based on Nagai's work (9). Peaks I and II, which had a higher apparent molecular weight than the collagenase, degraded the octapeptide model substrate to two peptides which had identical elution times in the HPLC assay to the product peptides produced by authentic collagenase (peak III). However, these peptidases did not degrade collagen. The HPLC data suggest that the site of cleavage in the octapeptide produced by the two peptidases is at the Gly–Ile bond. Other workers have shown the presence of high-molecular-weight peptidase activity from tadpole back-skin tissue culture medium (10), but the similarity in specificity to that of collagenase has not been heretofore demonstrated.

DISCUSSION

We have devised a simple, rapid, and sensitive means of quantitatively resolving by reverse-phase chromatography the two product peptides expected from the action of tissue collagenase on the model substrate DNP-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg. One product peptide cochromatographed with authentic DNP-Pro-Gln-Gly. The minimum detectable amount of this derivative in the system used here was about 0.1 nmol. This amount could be reduced by 10- to 50-fold by increasing the sensitivity of the detector without an unacceptable increase in noise level if a flat base line could be achieved. Since about 50 nmol of the peptide substrate is required for the spectrophotometric assay of Masui *et al.* (3), the use of HPLC results in a considerable savings in reagents and in enzyme.

A serious disadvantage in using the model peptide derivative to assay crude or impure systems for collagenase activity is the possible presence of peptidases which might cleave the model peptide, but which are de-

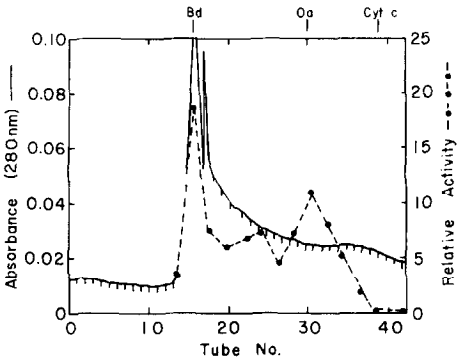

FIG. 7. Resolution of the 50% ammonium sulfate precipitate of lyophilized tadpole back-skin tissue culture medium into three fractions exhibiting collagenase-like specificity toward the synthetic substrate DNP-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg. Chromatography was carried out on a 2.5 × 40-cm column of Sephadex G-200 at 6°C. Fractions were assayed by incubation of 5-μl aliquots with 1.7 mM DNP-substrate octapeptide at pH 7.6, 37°C as described in the text. The elution position of Blue Dextran 2000 (Bd), ovalbumin (Oa) and cytochrome *c* (Cyt c) are shown at the top. Relative activity is defined as mm DNP-Pro-Gln-Gly peak height/min/ml enzyme solution. The pathlength of the uv monitor was 2 mm. Tubes 14–18, 19–27, and 28–36 were pooled and concentrated using Schleicher & Schuell Grade UH100/25 collodion bags in a vacuum filtration apparatus. These three pooled fractions constitute fractions I, II, and III referred to in the text.

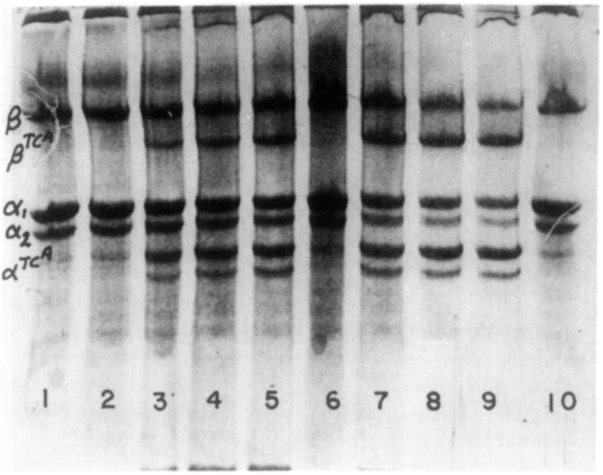

FIG. 8. Cleavage of acid-soluble calf skin collagen by fraction III (tubes 28–36 of Fig. 7). Unreacted collagen chains (β, α_1, and α_2 bands) were separated from cleaved chains (β^{TC^A}, $\alpha_1^{TC^A}$, and $\alpha_2^{TC^A}$) by electrophoresis in 6% polyacrylamide slab gels by the method of Laemmli (6). Lanes 1, 6, and 10 contained unreacted collagen alone; lanes 2–5 and 7–9 contained the collagen–collagenase reaction mixture at 0, 30, 60, 90, 120, 150, and 180 min after starting the reaction. Each well contained approximately 40 μg collagen. The TC^B fragments are the bottom bands in the gel. Other conditions are given in the text.

void of collagenolytic activity. Indeed, Masui *et al.* found exopeptidase activity in human serum which could be suppressed by including the sequence -Gln-D-Arg in the model substrate. As the data of Fig. 7 clearly demonstrate, unpurified culture medium from tadpole back skin contains endopeptidases which, as judged from the similarity between the HPLC elution patterns with authentic collagenase, cleave the model substrate at the Gly–Ile position. These endopeptidases are apparently different from those observed by Harper and Gross in tadpole tissue culture medium (10) which cleaved the model peptide phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-D-Arg since these enzymes were not precipitated by 50% ammonium sulfate. In addition, the apparent molecular weight of the peptidases of Harper and Gross were lower rather than greater than that of collagenase as observed in our studies.

Since peptidases without collagenase activity can give identical or similar cleavage patterns as authentic collagenases, it can be argued that such model compounds should not be used routinely to assay collagenases. However, there are certain instances where assay with the model peptide is acceptable. For example, if it is demonstrated that the ratio of collagenase to peptidase activity is constant throughout the purification procedure, then it is likely that noncollagenolytic peptidases are absent. The synthetic peptide assay may also be useful in mapping the active site of the enzyme and in providing a set of reproducible standard activities against which various purified collagenases from different sources in different laboratories might be compared. Finally, we have found the HPLC assay system is useful in evaluating several active site directed, metal binding peptide inhibitors of vertebrate collagenase which we have synthesized (11,12). In this case, we were able to monitor the stability of the synthetic inhibitors during the assay for collagenase activity since the inhibitor peptides could readily be separated from the product and substrate peptides by slight modifications of the elution program.

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REFERENCES

1. Woolley, D. E., and Evanson, J. M. (eds). (1980) Collagenase in Normal and Pathological Connective Tissues, Wiley, New York.
2. Gross, J., Harper, E., Harris, E., Jr., McCroskery, P., Highberger, J. H., Corbett, C., and Kang, A. H. (1974) *Biochem. Biophys. Res. Commun.* **61,** 605–612.
3. Masui, Y., Takemoto, T., Sakakibara, S., Hori, H., and Nagai, Y. (1977) *Biochem. Med.* **17,** 215–221.
4. Porter, R. R. (1957) *in* Methods in Enzymology (Colowick, S. P., and Kaplan, N. O., eds.), Vol. 4, p. 231, Academic Press, New York.
5. Nagai, Y., Lapiere, C. M., and Gross, J. (1966) *Biochemistry* **5,** 3123–3130.
6. Laemmli, U. (1970) *Nature (London)* **227,** 680–685.
7. Bradford, M. (1976) *Anal. Biochem.* **72,** 248–254.
8. Hancock, W. S., Bishop, C. A., Meyer, L. J., Harding, D. R. K., and Hearn, M. T. W. (1978) *J. Chromatogr.* **161,** 291–298.
9. Hori, H., and Nagai, Y. (1979) *Biochim. Biophys. Acta* **566,** 211–221.
10. Harper, E., and Gross, J. (1970) *Biochim. Biophys. Acta* **198,** 286–292.
11. Gray, R. D., Saneii, H. H., and Spatola, A. F. (1981) *Biochem. Biophys. Res. Commun.* **101,** 1251–1258.
12. Saneii, H. H., Gray, R. D., and Spatola, A. F. (1981) *in* Seventh American Peptide Symposium, Madison, Wisconsin, June 14–19, 1981, Abstr., p. 18.