Biochimica et Biophysica Acta, 571 (1979) 313—320

BBA 68871

SEPARATION OF COLLAGENASE AND A METAL-DEPENDENT ENDOPEPTIDASE OF RAT UTERUS THAT HYDROLYZES A HEPTAPEPTIDE RELATED TO COLLAGEN

J. FREDERICK WOESSNER, Jr.

Departments of Biochemistry and Medicine, University of Miami School of Medicine, Miami, FL 33152 (U.S.A.)

(Received April 10th, 1979)

Key words: Collagenase; Endopeptidase; Collagen heptapeptide

Summary

1. The synthetic peptide, 2,4-dinitrophenyl-L-Pro-L-Leu-Gly-L-Ile-L-Ala-Gly-L-Arg-amide (DNP-peptide) was tested as a potential substrate for uterine collagenase. Rat uteri were homogenized and the insoluble fraction was extracted at 60°C to obtain collagenase. The extracts were chromatographed on Sephadex G-150 to yield two peaks of DNP-peptide hydrolyzing activity. Peak I was completely inhibited by EDTA and had a molecular weight greater than 100 000. Peak II was inhibited about 90% by EDTA and had an apparent molecular weight of about 70 000.

2. Peak II coincided closely, but not exactly, with the peak of collagenase activity. It differed from collagenase in heat stability, binding properties on CM-Sephadex and failure to display latency.

3. Peak II represents a new endopeptidase activity. It has a pH optimum of 7 and it cleaves the DNP-peptide at the Gly-Ile and, possibly, the Leu-Gly bond.

4. The DNP-peptide is not a satisfactory substrate for the assay of impure collagenase preparations nor does it inhibit the action of collagenase on collagen substrate when added in 30-fold molar excess.

Introduction

The assay of animal collagenases presents a difficult problem. The substrate collagen is troublesome to prepare, there is often a high background of material digestible by nonspecific proteases such as trypsin, long incubations are required and the end products must be quantitated by separation from undigested collagen. The work of Nagai and coworkers [1,2], which suggested

that small synthetic peptides based on collagen sequences could serve as substrates for tadpole collagenase, promised an improved approach to assaying collagenase.

The peptide cleaved most rapidly by collagenase was 2,4-dinitrophenyl-L-Pro-L-Leu-Gly-L-Ile-L-Ala-Gly-L-Arg-amide [2]. This compound will be referred to by the abbreviation DNP-peptide. Although not completely specific for collagenase, this was the first synthetic peptide to be marketed by the Institute of Protein Research, Osaka, Japan. I endeavored to apply this substrate to the assay of collagenase extracted from the insoluble fraction of homogenates of rat uterus. It was hoped that nonspecific endopeptidases, if present, would be discarded with the soluble fraction. The further purification of collagenase in 60°C extracts of the insoluble fraction could then be accelerated by the convenient peptide assay. This plan was thwarted by the discovery that these extracts contained a second protease that cleaved the peptide, but was not a collagenase. The present paper describes some properties of this endopeptidase, which appears to be a new member of the metalloprotease class.

Methods

Enzyme extraction

Pregnant female rats were obtained from Sprague-Dawley, Madison, WI, U.S.A. The uteri were removed approximately 24 h after parturition and were frozen. Ref. 3 describes how the uteri were homogenized in 0.01 M $CaCl_2$/0.25% Triton X-100 and centrifuged to obtain a 6000 × *g* pellet. This pellet was then extracted by heating to 60°C for 4 min in 0.04 M Tris/HCl buffer, pH 7.4, containing 0.1 M $CaCl_2$ and 0.15 M NaCl [3]. Collagenase activity was assayed using fluorescein-labeled insoluble collagen prepared from rat tail tendon [3,4].

Peptide assay method

DNP-peptide (2,4-dinitrophenyl-L-Pro-L-Leu-Gly-L-Ile-L-Ala-Gly-L-Arg-amide) was purchased fron Peninsula Laboratories, Inc., San Carlos, CA, U.S.A. The analogous hexapeptide lacking the proline residue was a generous gift of Dr. S. Sakakibara, Inst. of Protein Research, Osaka, Japan. The peptide was dissolved at a final concentration of $4.1 \cdot 10^{-4}$ M, in 0.05 M Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl. The solution was distributed (100 μl/tube) to 3-ml conical centrifuge tubes with glass stoppers. These aliquots were frozen, lyophilized, and stored in the freezer until used. Exposure to light was minimized in all steps of preparation and assay.

For assay, 100 μl of enzyme preparation, adjusted to pH 7.5, were added to a peptide-containing tube and incubated at 37°C for 2 h. The reaction was stopped by adding 0.2 ml of 1 M HCl. Extraction of the digestion products into 1.4 ml ethyl acetate was accomplished by vortex mixing for 60 s followed by brief centrifugation. One ml of extract was transferred to a 1 ml cuvette with 1 cm pathlength, and the absorbtion was measured at 365 nm. Blanks were prepared by using buffer in place of enzyme, or by boiling the enzyme before assay. EDTA inhibition was determined by adding EDTA at three times the molarity of Ca^{2+} in the enzyme preparation. It was necessary to make the

EDTA solution slightly alkaline so that it did not lower the pH of the incubation mixture below 7.5 when mixed with calcium-containing solutions.

Enzyme chromatography

12-g batches of frozen uterus were carried through the procedure described above to obtain approximately 115 ml of extract. This was concentrated to 32 ml by membrane ultrafiltration (PM-10 membrane, Amicon Corp., Lexington, MA, U.S.A.). This and subsequent steps were carried out at 4°C. The concentrated sample was applied to a 2.6 × 90 cm column of Sephadex G-150 equilibrated with 0.04 M Tris-HCl/0.15 M NaCl/0.01 M $CaCl_2$, pH 7.5. The column was eluted with the same buffer at a rate of 20 ml/h.

Fractions containing collagenase activity were pooled from the G-150 separation and further chromatographed on a carboxymethyl-Sephadex column. CM-Sephadex C-50, 40-120 mesh, was equilibrated with 0.05 M sodium acetate buffer, pH 5. It was then packed in a 0.9 × 16 cm column under gravity flow. Since collagenase cannot tolerate this low pH, it was applied in a pH 6.0 buffer comprising 0.04 M Tris-HCl/0.01 M $CaCl_2$. Before the enzyme was added, the column was washed extensively with this same buffer until the effluent pH rose to pH 5.4. Approximately 60 ml of collagenase was equilibrated with this pH 6.0 buffer by extensive dialysis, then applied to the column. It was washed in with 12 ml of buffer, followed by 24 ml of 0.04 M Tris-HCl/0.01 M $CaCl_2$ at pH 8.0. This latter buffer was then made 0.1 M in NaCl and elution was continued with 16 ml of this solution. Finally, elution was concluded with a linear gradient of 50 ml 0.1 M NaCl and 50 ml 1.5 M NaCl in the pH 8.0 buffer.

Results

In early experiments, extracts of the insoluble fraction of rat uterine homogenates were chromatographed on Sephadex G-150 and the fractions containing collagenase activity were pooled. This pool was tested to determine its ability to digest the DNP-peptide. Positive results were obtained and the pools were then used to establish the validity of the assay system. Total digestion of the substrate gave a color yield of 0.41 absorbance units. A fixed amount of enzyme gave a linear response with increasing time up to 0.175 absorbance units or 40% hydrolysis of the substrate. When the assay time was fixed at 2 h, the assay was linear with increasing enzyme concentration up to 0.15 absorbance units. The final assay conditions settled upon were a 2-h incubation at 37°C and an amount of enzyme giving a change of 0.02—0.15 absorbance units.

Next, the assay was used to determine the distribution of DNP-peptide hydrolyzing activity across the elution pattern of the G-150 column. Fig. 1 illustrates the results. Two peaks were observed, the first emerges in the void volume and has an apparent molecular weight greater than 100 000. This accounts for 40% of the total activity recovered. The second peak lies almost directly under the collagenase peak. Several repeat analyses showed that this peak is consistently displaced 7—10 ml beyond the peak of collagenase activity. The collagenase (in latent form) has a mol. wt. of 77 000 [3]; using the same

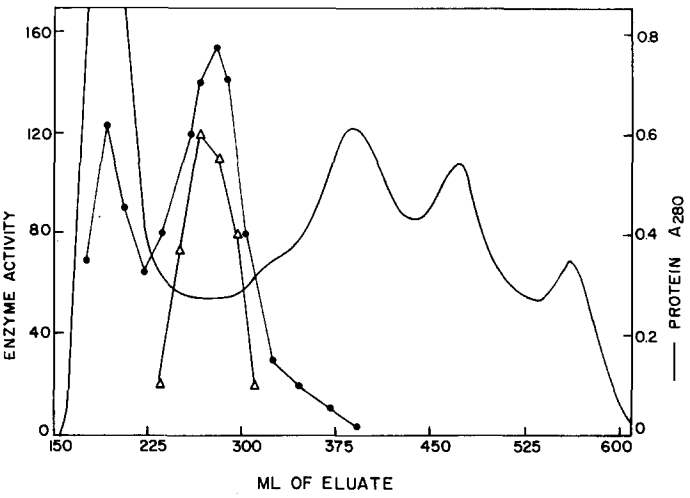

Fig. 1. Chromatography on Sephadex G-150 of a 60°C extract of rat uterine pellet. The conditions are described under Methods. ——— Protein based on absorption at 280 nm. ●———●, DNP-peptide hydrolysis, $A_{365} \times 10^3$ units/ml. △———△ collagenase activity measured by release of fluorescent peptides from insoluble collagen. This is total collagenase activity measured after activation by trypsin and addition of soybean trypsin inhibitor.

calibration curve, the DNP-hydrolyzing activity has an apparent mol. wt. of approx. 70 000. This second peak contains 60% of the activity. No lower molecular weight peak could be detected.

No further study was made of the first peak except to demonstrate that the activity was completely blocked by EDTA. Attention was directed to the second peak, since the concern was to determine if collagenase could cleave the DNP-peptide. Fractions between 240 and 300 ml were pooled for further study. This pool contained 0.01 M $CaCl_2$. When EDTA was added to a final concentrations of 0.03 M, the DNP-peptide hydrolyzing activity was inhibited to the extent of 85—92% in various preparations. Iodoacetate and *N*-ethylmaleimide at 10^{-4} M were not inhibitory, but phenylmethyl sulfonyl fluoride at this concentration produced a 10—15% inhibition. It appears that the major fraction of the activity in Peak II is metal-dependent. This is consonant with its being collagenase.

However, further study indicated that the major part of the DNP-peptide cleaving activity is not collagenase. In addition to the slight but consistent displacement of the peaks noted above, it was also found that different preparations did not give a constant ratio of DNP-peptide/collagen digestion. More striking was the fact that most of the collagenase activity was in a latent form, but none of the DNP-peptide cleaving activity has even been found to be latent. Latent enzyme is activated by incubating 1 ml fractions with 10 μg of trypsin for 10 min at 37°C, followed by the addition of 36 μg soybean trypsin inhibitor [3]. Heating the enzyme preparation at 60°C for 15 min resulted in the loss of 40% of the DNP-peptide hydrolyzing activity, while only 3% of the collagenase activity was lost. By 30 min the respective values were 78% and 13%. This clearly points to the presence of two different enzymes. The enzyme pool

from G-150 was further resolved by chromatography on CM-Sephadex (Fig. 2). About 85% of the DNP-peptide hydrolyzing activity fails to stick to the column and emerges directly, while only 7% of the collagenase activity emerges in this region. Less than 10% of the DNP-peptide cleaving activity was lost during chromatography. It appears that the major portion of the DNP-peptide hydrolyzing activity does not follow collagenase during this chromatography. The trace of collagenase activity under this first peak has not been proved to be a true collagenase, it could just reflect the release of small amounts of fluorescent telopeptides from the insoluble collagen substrate. On the other hand, the collagenase peak still displays significant activity against the DNP-peptide, so an action of collagenase on this substrate cannot be excluded. However, it can be concluded that the major portion of DNP-peptide hydrolyzing activity in the uterus is recoverable in two major peaks on G-150, neither of which corresponds to the major collagenase peak.

Further properties of the DNP-peptide hydrolyzing activity in the G-150 pool have been established. Fig. 3 shows that the pH optimum is about 7.0. Activity was tested against the hexapeptide DNP-L-Leu-Gly-L-Ile-Ala-Gly-L-Arg-NH_2. The rate of cleavage in two experiments was approximately 20% of that observed with the heptapeptide. The specificity of cleavage of the heptapeptide was studied by drying down the ethyl acetate extracts of an extensively-digested (75%) sample. This was hydrolyzed under N_2 in 6 M HCl and subjected to amino acid analysis on a Durrum D-500 analyzer (courtesy of Dr. K. Brew, Department of Biochemistry). A small amount of heptapeptide is extracted into ethyl acetate and this was reflected in the presence of small amounts of all the amino acids. Only Pro, Leu and Gly were found in excess amount, indicating a split at the Gly-Ile bond as originally reported by Masui et al. [2]. However, Gly was present only to the extent of one-third of the Leu content, indicating that there is either a second, and major, point of cleavage at the Leu-Gly bond, or that Gly is removed by subsequent carboxypeptidase action.

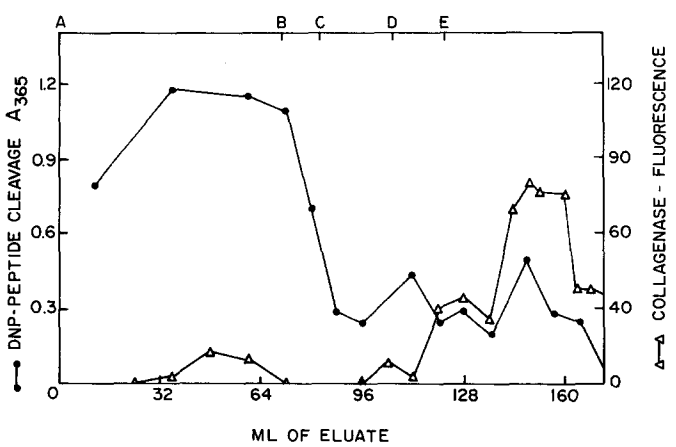

Fig. 2. Chromatography of enzyme pool from G-150 on CM-Sephadex. Details are given under Methods. Letters indicate: A, enzyme application; B, wash at pH 6; C, buffer change to pH 8; D, addition of 0.1 NaCl; E, start gradient. •, DNP-peptide hydrolyzing activity; △, collagenase activity.

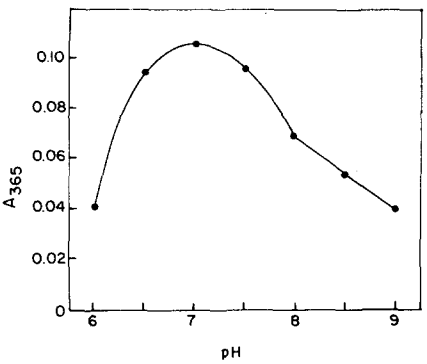

Fig. 3. pH curve for the digestion of DNP-peptide by G-150 enzyme pool. Tris buffer, 2 M, was adjusted to various pH values so that when added to 10 vols. of buffer from the G-150 column, the final pH would be the values indicated on the figure. The final Tris concentration during the assay was 0.29 M.

This analysis was done only once, and the quantitative aspect should not be overinterpreted due to the impure state of the enzyme, but it is clear that the enzyme activity is of the endopeptidase type.

Efforts were made to detect the DNP-peptide hydrolyzing activity in pellets of involuting rat uterus prior to extraction at 60° C. This has been done successfully in the case of collagenase; indeed, labeled collagen can be stirred in with resuspended pellets and will undergo digestion [5]. However, when DNP-peptide was added to the pellets, no release of ethyl-acetate extractible color could be detected after 18 h incubation. Perhaps the enzyme activity is inhibited by a tissue factor. An attempt was made to inhibit the digestion of collagen in the pellet by adding excess peptide. Collagen digestion was measured by the release of hydroxyproline as previously described [3]. Collagen concentration in the pellet assay was $1.3 \cdot 10^{-5}$ M. DNP-peptide was added at levels from $1.3 \cdot 10^{-5}$ to $3.5 \cdot 10^{-4}$ M with no detectable effect on the rate of collagen digestion.

DNP-peptide hydrolyzing activity was not sought in the soluble supernatant fraction of uterine homogenates, but it could be detected in the 60° C extracts of the insoluble pellets prior to chromatography on G-150. 1 g of wet tissue yielded sufficient enzyme to digest $2.9 \cdot 10^{-7}$ mol of peptide/h when the peptide concentration was $4.1 \cdot 10^{-4}$ M. For comparison, the corresponding amount of collagenase was $1 \cdot 10^{-9}$ mol collagen digested/h when the collagen concentration was $7.1 \cdot 10^{-6}$ M.

Discussion

The original paper of Masui et al. [2] indicated that collagenase from tadpole tissue cleaved the DNP-heptapeptide at a rapid rate, but that this peptide was not completely specific for collagenase. More recently, Kobayashi and Nagai [6] studied a more specific peptide: DNP-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg. They found that extracts of human leukocytes digested this peptide and that chromatography on Sephadex G-200 gave three peaks of activity. Peak I had a mol. wt. of 150 000 and also showed gelatinase activity. Peak II had a mol. wt.

of 75 000 and coincided with collagenase. Peak III had a mol. wt. of 25 000 and was a serine protease. Peaks I and II were both metal-dependent. Seltzer et al. [7] have observed an enzyme in fibroblast culture media which cleaves this same DNP-peptide and gelatin and has a mol. wt. of 120 000. Their enzyme may be related to Peak I of Kobayashi and Nagai [6]. The rat uterus extract gives two peaks of metal-dependent activity which bear at least a superficial resemblance to two of these other enzymes that have been reported.

The present paper is the first to explore in detail the endopeptidase activity that coincides with the collagenase peak and has a mol. wt. of approx. 70 000. It is clear that the major part of this activity against DNP-peptide does not coincide with the collagenase activity in any of its properties except metal-dependency and molecular size. It is merely fortuitous that both activities coelute from G-150. In addition to the small difference in molecular weight, the two enzymes also differ in heat stability, affinity for CM-Sephadex, and occurrence as latent forms. It is clear that the peptide substrate is not suitable for the assay of collagenase (even if one uses a more specific peptide [6]), at least not at the present stage of collagenase purification. In fact, the present study gives rise to the question of whether the small peptides are even susceptible to collagenase action. Perhaps collagenase preparations examined to date have contained trace amounts of endopeptidase activity. The uterine collagenase has not been purified sufficiently to test this point, but other preparations could be tested by heating to 60°C to destroy these traces selectively.

The molecular weight and metal dependency of the uterine endopeptidase are reminiscent of PZ-peptidase (peptidase for 4-phenylazobenzyloxycarbonyl-L-Pro-L-Leu-Gly-L-Pro-D-Arg) [8]. However, in preliminary studies it has not been possible to detect any cleavage of the substrate 4-phenylazo-benzyloxy-carbonyl-L-Pro-L-Leu-Gly-L-Pro-D-Arg by fractions from the Sephadex G-150 column. The PZ-peptidase activity previously reported in rat uterus [9] was found in the supernatant of the first homogenate and was more soluble than the present endopeptidase.

In addition to collagenase, several other metal-dependent proteases have recently been found in connective tissue sources, including gelatinase [10] and proteoglycanase [11]. All three of these enzyme activities show two common properties: inhibition by EDTA and latency that can be overcome by trypsin activation. The uterine endopeptidase is distinctive in failing to display latency. Each of the other proteases found so far has a clear role to play in the degradation of connective tissue; the role of the present enzyme is not yet clear. The Gly-Ile bond occurs only three times in the collagen chain [12], however, Leu-Gly bonds are much more frequent. Further purification must be accomplished before a definite answer can be found to this question.

Acknowledgements

The author would like to thank Mrs. Carolyn Taplin for her excellent technical assistance. Mr. Thomas Murray also assisted in the early phases of this study. This research was supported by a grant from the National Institute of Child Health and Human Development, N.I.H., HD-06773.

References

1 Nagai, Y., Masui, Y. and Sakakibara, S. (1976) Biochim. Biophys. Acta 445, 521—524
2 Masui, Y., Takemoto, T., Sakakibara, S., Hori, H. and Nagai, Y. (1977) Biochem. Med. 17, 215—221
3 Woessner, Jr., J.F. (1977) Biochem. J. 161, 535—542
4 Steven, F.A., Torre-Blanco, A. and Hunter, J.A.A. (1975) Biochim. Biophys. Acta 405, 188—200
5 Weeks, J.G. (1973) Collagenase in the Postpartum Rat Uterus, Doctoral Dissertation, University of Miami, FL
6 Kobayashi, S. and Nagai, Y. (1978) J. Biochem. 84, 559—567
7 Seltzer, J.L., Adams, S., Grant, G., Jeffrey, J.J. and Eisen, A.Z. (1979) Abst. 9th Southern Conn. Tissue Soc. Meet., New Orleans, p. 6
8 Morales, T.I. and Woessner, Jr., J.F. (1977) J. Biol. Chem. 252, 4855—4860
9 Ryan, J.N. and Woessner, Jr., J.F. (1972) Biochem. J. 127, 705—713
10 Sellers, A., Reynolds, J.J. and Meikle, M.C. (1978) Biochem. J. 171, 493—496
11 Huybrechts-Godin, G. and Vaes, G. (1978) FEBS Lett. 91, 242—245
12 Fietzek, P.P. and Kuehn, K. (1976) in Int. Rev. Connective Tissue Res. (Hall, D.A. and Jackson, D.S., eds.), Vol. 7, p. 1, Academic Press, New York