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Proteinases in Human Polymorphonuclear Leukocytes

Purification and Characterization of an Enzyme which Cleaves Denatured Collagen and a Synthetic Peptide with a Gly-Ile Sequence

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Polymorphonuclear leukocytes have been shown to contain proteolytic enzymes which are capable of degrading connective tissue proteins such as native collagen. In this study, proteolytic enzymes were extracted from human polymorphonuclear leukocytes and a neutral proteinase was extensively purified and characterized. The activity of this enzyme was monitored by degradation of denatured [^{3}H]proline-labeled type I collagen or by cleavage of a synthetic dinitrophenylated peptide with a Gly-Ile sequence. The enzyme was readily separated from leukocyte collagenase by concanavalin-A – Sepharose affinity chromatography and further purified by QAE-Sephadex ion-exchange chromatography and gel filtration on Sephacryl S-200. The purified enzyme had a molecular weight of approximately 105000, its pH optimum was about 7.8, and it was inhibited by Na_2EDTA and dithiothreitol, but not by fetal calf serum. The enzyme degraded genetically distinct type I, II, III, IV and V collagens, when in a non-helical form, but not when in native triple-helical conformation. Dansyl-monitored end-group analyses, combined with digestion by carboxypeptidase A, indicated that the enzyme cleaved denaturated type I collagen at Gly-Xaa sequences, in which Xaa can be leucine, isoleucine, valine, phenylalanine, lysine, or methionine. Thus, the purified enzyme referred to here as Gly-Xaa proteinase, is a neutral proteinase, which may be of importance in inflammatory disease processes by degrading further collagen peptides which have been rendered non-helical as a result of collagenase cleavage.

Collagen, the major fibrillar component of most connective tissues, has a unique triple-helical conformation which confers several unusual properties on this protein molecule. In particular, this structure is remarkably resistant to a variety of proteolytic enzymes, and the degradation of collagen *in vivo* may be initiated only by specific metalloproteinases, collagenases [1–3]. The vertebrate collagenases degrade collagen only at a specific region located at three-quarters of the length of the molecule from the amino terminus; the cleavage of type I, II, and III collagens has been shown to occur at Gly-Leu or Gly-Ile sequences [4, 5]. Specific collagenolytic enzymes have been demonstrated in several human cells and tissues, including polymorphonuclear leukocytes, macrophages, and fibroblasts, as well as skin and synovium explants in culture [2, 6]. In addition, specific collagenases degrading 'non-interstitial' collagens, types IV and V, have been demonstrated [7–14].

A preliminary report of this work has been presented at the Western Regional Meeting of the American Federation for Clinical Research, Carmel, California, February, 1982 [Ryhänen, L., Rantala-Ryhänen, S., and Uitto, J. (1982) *Clin. Res. 30*, 29A].

Abbreviations. Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Cl_3AcOH, trichloroacetic acid; $PhMeSO_2F$, phenylmethylsulfonyl fluoride; MalNEt, *N*-ethylmaleimide; OH-HgBzOH, *p*-hydroxymercuribenzoate; Dns, dansyl; Me_2SO, dimethylsulfoxide; Bes, 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-glycine; SDS, sodium dodecyl sulfate; DNP-peptide, 2,4-dinitrophenyl-L-prolyl-L-glutaminyl-L-glycyl-L-isoleucyl-L-alanyl-L-glycyl-L-glutaminyl-D-arginine.

Enzymes. Carboxypeptidase A (EC 3.4.17.1); α-chymotrypsin (EC 3.4.21.1); collagenase (EC 3.4.24.3); pepsin (EC 3.4.23.1); trypsin (EC 3.4.21.4); Gly-Xaa proteinase (EC 3.4.99.–).

After the initial cleavage by a collagenase, the degradation products of the triple-helical collagen molecule undergo rapid helix-to-coil transformation at normal body temperatures. Although the non-helical polypeptides are susceptible to proteolysis by several proteolytic enzymes, it has been suggested that the degradation of collagen *in vivo* to small peptides and free amino acids involves enzymes specific to denatured collagen; these enzymes have been called 'gelatinases' [6]. An enzyme apparently specific to denatured collagen has been recently purified from human skin explant cultures [15], and similar enzymatic activity has been demonstrated in human polymorphonuclear leukocytes [16, 17].

In the present study, we have extensively purified from human polymorphonuclear leukocytes a proteinase which cleaves denatured type I, II, III, IV and V collagens and a synthetic peptide with a Gly-Ile sequence. This enzyme does not cleave the same collagens in native conformation, and it can be separated from the polymorphonuclear leukocyte collagenase by affinity chromatography on concanavalin-A – Sepharose. The enzyme cleaves most actively Gly-Ile and Gly-Leu peptide sequences in type I collagen, but cleavage at other Gly-Xaa sequences can also be demonstrated: therefore, this enzyme is designated here as Gly-Xaa proteinase.

MATERIALS AND METHODS

Materials

L-[2,3-^{3}H]Proline (specific activity 27 Ci/mmol) was purchased from New England Nuclear. Pepsin (twice crystallized), trypsin, α-chymotrypsin and soybean trypsin inhibitor were obtained from Sigma. Fetal calf serum was purchased

from Gibco. Bacterial collagenase, chromatographically purified, was provided by Elastin Products (St Louis).

Substrates

DNP-peptide, a synthetic peptide with the sequence 2,4-dinitrophenyl-L-prolyl-L-glutaminyl-L-glycyl-L-isoleucyl-L-alanyl-L-glycyl-L-glutaminyl-D-arginine [18] was purchased from Calbiochem.

For preparation of radioactively labeled type I collagen, 17-day-old chick embryo tendons were incubated for 4 h at 37 °C with [^{3}H]proline in the presence of 50 µg/ml 2-aminopropionitrile · HCl, and 2% dialyzed fetal calf serum in modified Krebs' medium, buffered with 30 mM Hepes, pH 7.6 [19]. Soluble [^{3}H]proline-labeled type I collagen, which was used as substrate for enzyme assays, was isolated by a procedure described previously [20]. When incubated with highly purified bacterial collagenase, 99% of the radioactivity in type I ^{3}H-labeled collagen preparation was converted to Cl_3AcOH-soluble peptides. Also, SDS/polyacrylamide slab gel electrophoresis of the prepared substrates [21] indicated that essentially all of the radioactivity was in α1(I) and α2(I) chains of type I collagen. The specific radioactivity of the type I [^{3}H]proline-labeled collagen was 3.9×10^7 counts min^{-1} mg^{-1}.

Unlabeled type I, III, and V collagens were isolated from human placenta by pepsin solubilization and differential salt precipitations [22]. Type II collagen was prepared from chick embryo sterna as described elsewhere [19]. The preparations of type I, II, III, and V collagens were judged to be essentially pure collagen by amino acid analysis. SDS/polyacrylamide slab gel electrophoresis, with and without reduction with 2-mercaptoethanol, indicated that the type I, II, and III collagen preparations consisted of α1(I) plus α2(I), α1(II), and α1(III) chains, respectively. The type V collagen preparation consisted mostly of α1(V) and α2(V) chains, but a small amount of type I collagen was also detected.

Unlabeled type IV collagen, purified from human placental tissues by pepsin solubilization and differential salt precipitation, was kindly provided by Dr Robert E. Burgeson (Department of Pediatrics, Harbor-UCLA Medical Center). The preparation consisted of type IV collagen, based on amino acid analysis and precipitation by a specific anti-(type IV collagen) antibody. On SDS/polyacrylamide gel electrophoresis, following reduction with 5% 2-mercaptoethanol, three distinct bands with apparent molecular weights of 170000, 140000 and 70000 were noted. In addition, large-molecular-weight aggregates, which did not penetrate 6% polyacrylamide gel, were present.

Separation of Collagenase and Gly-Xaa Proteinase

For purification of polymorphonuclear leukocyte proteinase, buffy coats of human blood were obtained from the Finnish Red Cross. The polymorphonuclear leukocytes were isolated by dextran sedimentation, as described previously [23]. The cells were homogenized in 50 mM Tris/HCl, pH 7.8, containing 0.2 M NaCl, 10 mM $CaCl_2$, and 0.1% Triton X-100, using a Teflon/glass homogenizer. The homogenate was frozen and thawed five times, and deoxyribonuclease (type I, chromatographically purified from bovine pancreas; Sigma) was added to a final concentration of 10 µg/ml. The sample was then incubated for 10 min at 25 °C. The homogenate was centrifuged at $10000 \times g$ for 30 min, and the supernatant was chromatographed at 4 °C on a concanavalin-A–Sepharose column (2.5 × 20 cm), equilibrated with 50 mM Tris/HCl, pH 7.8, containing 0.2 M NaCl and 10 mM $CaCl_2$. The column, after application of the sample, was washed with the same buffer, and then eluted with a buffer containing 0.5 M methyl α-D-mannopyranoside or 0.5 M methyl α-D-glucopyranoside. The fractions were analyzed for proteinase activity using DNP-peptide and native or denatured type I collagen as substrate, as described below, and in Fig. 1.

Essentially, all collagenase activity was detected in the fraction which did not bind to concanavalin-A–Sepharose (Fig. 1). This collagenase activity was further through several chromatographic steps; the details of the purification will be reported elsewhere. The partially purified collagenase was used in the present study, as indicated in the text.

Purification of Gly-Xaa Proteinase

After concanavalin-A–Sepharose chromatography (Fig. 1), the fractions containing proteolytic activity degrading denatured type I collagen and DNP-peptide were pooled, fractionated by ammonium sulfate precipitation (30–80% saturation) and purified further at 4 °C, as follows. The ammonium sulfate precipitate was dissolved in 50 mM Tris/HCl, pH 7.8, containing 0.2 M NaCl and 10 mM $CaCl_2$, dialyzed against the same buffer, and chromatographed on Sephacryl S-200, equilibrated and eluted with 25 mM Tris/HCl, pH 8.0, containing 50 mM $CaCl_2$. The fractions containing the enzyme activity were pooled, diluted by the addition of an equal volume of 25 mM Tris/HCl, pH 8.0, containing 5 mM $CaCl_2$, and chromatographed on a QAE-Sepharose A-25 ion-exchange column (1.5 × 50 cm). The column was washed with 25 mM Tris/HCl, pH 8.0, containing 25 mM NaCl and 5 mM $CaCl_2$, and the proteins were then eluted with a gradient from 25 mM NaCl to 300 mM NaCl in 25 mM Tris/HCl, pH 8.0, containing 5 mM $CaCl_2$ (Fig. 2). The fractions containing the enzyme activity were pooled, concentrated on Amicon PM-30 ultrafiltration membranes, and finally chromatographed on a Sephacryl S-200 gel filtration column, equilibrated and eluted with 50 mM Tris/HCl, pH 7.8, containing 1 M NaCl and 10 mM $CaCl_2$ (Fig. 3). The purified enzyme protein was concentrated on the Amicon PM-30 ultrafiltration membrane, and studied as described in Results.

Assay Procedures

The enzymatic degradation of DNP-peptide was determined as described by Masui et al. [18]. In this procedure, the enzymatic digestion of the substrate was terminated by the addition of 2.5 vol. of 1 M HCl. The amino-terminal degradation fragment containing the dinitrophenyl group was extracted into ethyl acetate, and the absorbance of the organic layer measured at 365 nm.

To assay the proteinase activity degrading denatured collagen, aliquots of radioactive collagen substrate (0.15–1.5×10^5 counts/min per sample) were incubated in 50 mM Tris/HCl, pH 7.8, containing 0.2 M NaCl, 10 mM $CaCl_2$, 10 mM MalNEt, and 20 µg/ml bovine serum albumin (Sigma) in a final volume of 100 µl. The collagen used as substrate was denatured by heating at 100 °C for 10 min and then rapidly cooled to 0 °C prior to assay. The incubations were performed at 37 °C, usually for 30 min. The enzyme digestion was terminated by the addition of Na_2EDTA to a final concentration of 40 mM, and the sample was cooled to 0 °C. Soluble unlabeled type I carrier collagen, prepared as described elsewhere [24], was added to a final concentration of

0.4 mg/ml. An equal volume of ice-cold 20% Cl_3AcOH (trichloroacetic acid) was then added, and after a 20-min incubation at 4 °C, the Cl_3AcOH precipitates were filtered on a manifold using glass-fiber filters (Millipore AP 1502500). The filters were washed with 10% Cl_3AcOH, extracted in 10 ml of scintillation fluid (3a70B; Research Products International Corp.) and counted in a Beckman LS 7500 liquid scintillation counter. The counting efficiency of ^3H-labeled substrate recovered in the filters was 36% [20]. In each assay, control samples without added enzyme were incubated and processed in a parallel manner. The enzyme activity was expressed as the difference in ^3H radioactivity between the controls incubated without the added enzyme and the test samples containing the proteinase activity.

The collagenase activity was determined by incubation with native type I ^3H-labeled collagen ($0.15-1.5\times10^5$ counts/min per sample) at 37 °C as described elsewhere [20]. The degradation products were assayed by a brief proteolytic digestion which a mixture of trypsin and α-chymotrypsin, employing conditions under which uncleaved collagen molecules resist proteolysis but the collagenase cleavage products, TC^A and TC^B, are digested to Cl_3AcOH-soluble peptides [20, 25, 26]. The Cl_3AcOH-soluble peptides were assayed by filtration as described below.

SDS/Polyacrylamide Slab Gel Electrophoresis

The degradation of collagen substrates was also followed by SDS/polyacrylamide slab gel electrophoresis. Incubations were terminated by the addition of $PhMeSO_2F$ (phenylmethylsulfonyl fluoride; final concentration 3 mM), MalNEt (*N*-ethylmaleimide; 10 mM), and Na_2EDTA (20 mM). The samples were then heated at 100 °C for 10 min with 4 M urea, 2% SDS, 0.001% bromphenol blue, 10% glycerol, and 0.125 M Tris/HCl, pH 6.8. To some samples, 5% 2-mercaptoethanol was added. The samples were electrophoresed according to King and Laemmli [27]. Radioactive proteins were visualized by fluorography [21]; unlabeled protein bands were visualized by staining with Coomassie blue.

Determination of Cleavage Sequences

To characterize the cleavage site(s) by the enzyme, DNP-peptide was incubated with enzyme preparation. The reaction mixture was then heated at 100 °C for 10 min, and cooled to 0 °C. An equal volume of 5% Cl_3AcOH was added to precipitate the enzyme protein. The sample was incubated at 4 °C for 60 min and then centrifuged at 2500 rev./min for 10 min. The supernatant containing the uncleaved DNP-peptide as well as the degradation products was then analyzed by thin-layer chromatography on silica gel G plates (20 × 20 cm). The chromatograms were developed in a solvent system of toluene/2-chloroethanol/pyridine/25% ammonia (50:35:15:7) [28]. The chromatogram was dried in a stream of cool air, and redeveloped in the same dimension using the same solvent system. Following chromatography, peptides containing the dinitrophenyl group could be visualized directly. The peptides devoid of the group were visualized by spraying with ninhydrin.

In a separate set of experiments, the DNP-peptide cleavage fragments were extracted in ethyl acetate. The organic solvent containing the cleaved dinitrophenylated fragment of the peptide, was evaporated and the residue hydrolyzed in 6 M HCl at 110 °C for 24 h. Amino acids were analysed using a Beckman 119CL amino acid analyzer.

To determine the amino-terminal amino acids in the cleavage products of type I collagen, samples (after incubation with an enzyme preparation) were adjusted to pH 10 and mixed with an equal volume of acetone containing dansyl chloride (Sigma), 1 mg/ml, and incubated for 60 min at 37 °C. The reaction was stopped by the addition of an equal volume of 12 M HCl. The samples were hydrolyzed for 24 h at 110 °C, filtered through a Millipore Millex-6S filter and evaporated to dryness under nitrogen.

To determine the carboxy-terminal amino acids in the cleavage products of type I collagen, samples (following incubation with the enzyme as above) were further incubated with 125 units/ml carboxypeptidase A (Sigma, type I) at 37 °C. After 10-min, 60-min or 180-min incubation, the samples were treated with dansyl chloride, as described above.

Dansylated amino acids were separated by two-dimensional polyamide sheet thin-layer chromatography (Baker-flex polyamide sheets, Baker Chemical, 10 × 10 cm) [29]. Chromatograms were developed in a solvent system consisting of 88% formic acid/distilled water (2:100); the sheets were dried in a stream of air and rechromatographed in the same direction with the same solvent system. After drying, the sheets were turned 90°, and developed in benzene/glacial acetic acid (90:10). Dansylated amino acids were visualized with an ultraviolet lamp at 254 nm; they were identified on the basis of their mobilities in relation to standard dansylated amino acids.

RESULTS

Purification of Gly-Xaa Proteinase

Buffy coats of human polymorphonuclear leukocytes were used as starting material for purification of proteolytic enzymes. Several different methods were employed to assay the proteolytic activities. First, native radioactive type I collagen was used as substrate for collagenase, and the enzymatic activity was determined by subsequent proteolysis of the cleavage products, as previously described by us [20]. Secondly, denatured type I collagen was used as substrate for the proteinase and proteolysis was monitored by conversion of the polypeptides into Cl_3AcOH-soluble fragments. Thirdly, a synthetic peptide (DNP-peptide) with the sequence 2,4-dinitrophenyl-L-prolyl-L-glutaminyl-L-glycyl-L-isoleucyl-L-alanyl-L-glycyl-L-glutaminyl-D-arginine, was used as a substrate; the cleavage was assayed as described by Masui et al. [18].

After initial extraction, as described in Materials and Methods, soluble proteins were chromatographed on a concanavalin-A–Sepharose affinity column (Fig. 1). When the proteolytic activity was assayed by the DNP-peptide substrate, three major peaks of activity were detected: the first peak (Fig. 1) did not bind to the column and eluted in the initial fractions 1–10; the second peak of activity, fractions 33–40, bound to the column and could be eluted with methyl α-glucoside; a small additional peak of activity could be eluted with methyl α-mannoside (fractions 44–46). The first peak always contained considerably less activity than the second peak towards DNP-peptide as substrate. When the proteolytic activity was measured using native type I collagen as substrate, essentially all of the activity was found in the first peak while the second and third peaks did not show any collagenolytic activity (Fig. 1). In contrast, if denatured type I collagen was used as substrate, no degradation was

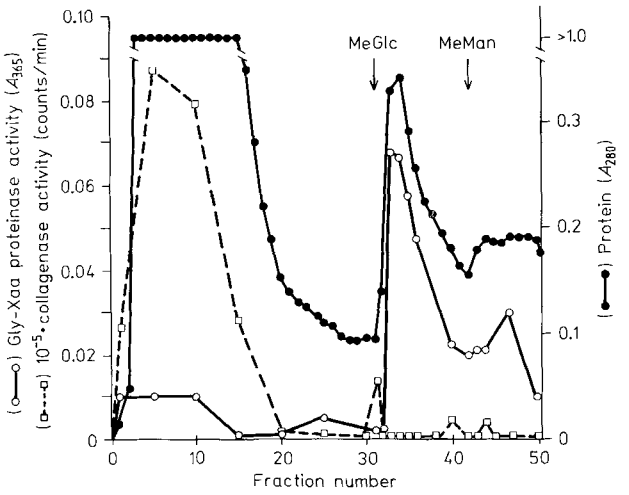

Fig. 1. *Separation of Gly-Xaa proteinase and collagenase on a concanavalin-A – Sepharose affinity column.* An homogenate of human polymorphonuclear leukocytes was chromatographed on concanavalin-A – Sepharose, as described in Materials and Methods. The flow rate was 5 ml/h and 5-ml fractions were collected. Protein concentration was monitored by absorbance at 280 nm. The Gly-Xaa proteinase activity was measured using DNP-peptide with a Gly-Ile sequence as substrate, and the activity of collagenase was assayed by incubation with native ^{3}H-labeled type I collagen, as indicated in Materials and Methods. The proteins initially bound to the column were eluted by 0.5 M methyl α-glucoside (MeGlc) and methyl α-mannoside (MeMan) as indicated by the arrows. Fractions 33 – 49 containing the Gly-Xaa proteinase activity were pooled and subjected to further purification. (●——●) Absorbance at 280 nm; (□– –□) collagenase activity; (○——○) enzyme activity measured with DNP-peptide as substrate

Table 1. *Purification of Gly-Xaa proteinase from human polymorphonuclear leukocytes*
The enzyme preparations were incubated with DNP-peptide as substrate; one unit of activity is defined as the amount catalyzing the release of 1 A_{365} unit of DNP-containing cleavage fragment by a 30-min incubation at 37 °C and which was subsequently extracted by ethyl acetate, as described in the text

Purification step	Total protein	Specific activity	Purification	Total activity	Yield
	mg	units/mg protein	n-fold	units	%
Homogenate	5400	0.14	1	756	100
Concanavalin-A – Sepharose	872	0.60	4	523	69
Ammonium sulfate precipitation (30 – 80%)	196	0.80	6	157	21
Sephacryl S-200 (in 0.05 M NaCl)	11.6	6.07	43	70	9
QAE-Sephadex	3.4	12.28	88	42	6
Sephacryl S-200 (in 1.0 M NaCl)	0.4	78.46	560	31	4

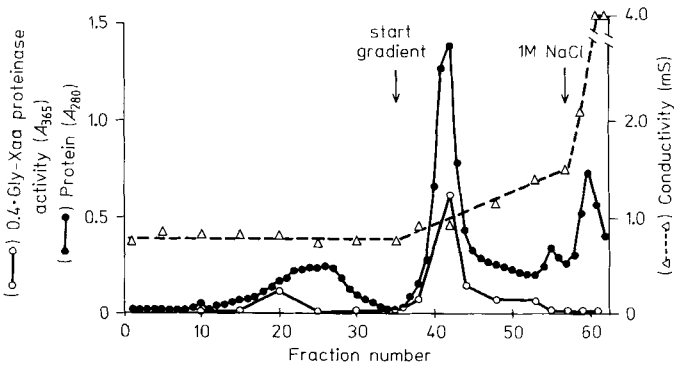

Fig. 2. *QAE-Sephadex ion-exchange chromatography of partially purified Gly-Xaa proteinase.* The enzyme protein eluted from a concanavalin-A – Sepharose column (Fig. 1) was further purified by ammonium sulfate precipitation and Sephacryl S-200 gel filtration chromatography, as described in the text. The sample was then chromatographed on QAE-Sephadex; the flow rate was 100 ml/h and 3-ml fractions were collected. The protein initially bound to the column was first eluted with a linear gradient of 25 – 300 mM NaCl and then with 1 M NaCl. Fractions 39 – 42 were pooled and analyzed further. (●——●) Absorbance at 280 nm; (○ —○) enzyme activity measured with DNP-peptide as substrate; (△– –△) conductivity

noted with the enzyme preparation eluting in the first peak while significant activity was found in the second peak (see below).

The proteolytic activity in the second peak, eluted from concanavalin-A – Sepharose, was further purified by consecutive steps consisting of ammonium sulfate fractionation, gel filtration chromatography on Sephacryl S-200 in 0.05 M NaCl, QAE-Sephadex ion-exchange chromatography, and a final gel filtration on Sephacryl S-200 in 1.0 M NaCl (Table 1). The proteolytic enzyme, using DNP-peptide as substrate, was purified 560-fold, and the overall recovery of the enzyme activity was 4%.

Characteristics of the Enzyme Protein

Examination of the eluate after QAE-Sephadex chromatography revealed that the majority of the enzymatic activity was contained in a single protein peak (Fig. 2). Subsequent chromatography of the enzyme protein on Sephacryl S-200 in the presence of 1 M NaCl yielded one major protein peak which coincided with the enzyme activity and which was separated from several contaminant proteins (Fig. 3). The estimated molecular weight of the active enzyme, in comparison with globular standard proteins, was 105000 (Fig. 3, insert). SDS/polyacrylamide gel electrophoresis of the protein peak coinciding with the enzyme activity revealed two closely migrating protein bands, each with an approximate molecular weight of 100000. Attempts to separate these proteins further by chromatofocusing were unsuccessful since the pI of both proteins appeared to be less then 4.0. This observation is consistent with preliminary amino acid analyses of the purified enzyme protein; the enzyme was rich in aspartic acid, serine and threonine, while relatively poor in arginine and lysine; hydroxyproline and hydroxylysine were not present.

Catalytic Properties of the Enzyme

For the determination of pH optimum, DNP-peptide and denatured type I collagen were used as substrates for the enzyme purified through the chromatographic steps depicted above. For both substrates, the pH optimum was about 7.8 (Fig. 4). However, very little difference was noted between pH values 7.1 and 8.5. Less than 50% of the maximum activity was noted below pH 6.0 and above pH 9.5 (Fig. 4).

To characterize the enzyme activity further, several known proteinase inhibitors and activators were tested, first using

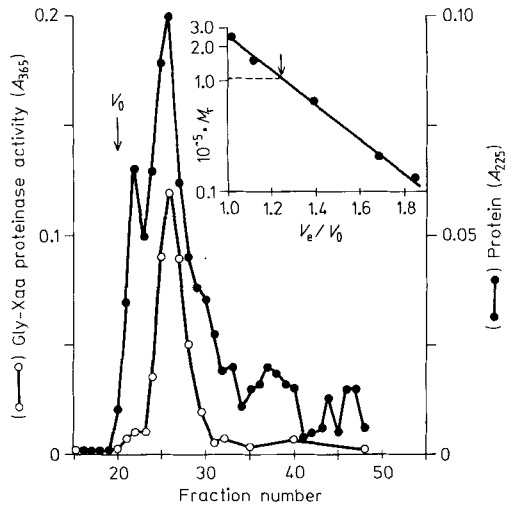

Fig. 3. *Gel filtration chromatography of partially purified Gly-Xaa proteinase on Sephacryl S-200.* The enzyme protein eluted from the QAE-Sephadex column (Fig. 2) was concentrated by ultrafiltration on an Amicon PM-30 membrane and applied to the gel filtration column; 3-ml fractions were collected. The protein concentration was monitored by absorbance at 225 nm, and the enzyme activity was determined by incubation with DNP-peptide as substrate. The following standards (with molecular weights) were used for calibration: blue dextran ($> 2.0 \times 10^6$), aldolase (1.8×10^4), bovine serum albumin (6.7×10^4), soybean trypsin inhibitor (20.1×10^3), ribonuclease A (13.7×10^3). Comparison with these standards gave an estimated molecular weight of 105000 for the Gly-Xaa proteinase (arrow in inset). Fractions 24–28 were pooled and the purified enzyme was subjected to further analyses. (●——●) Absorbance at 225 nm; (○——○) enzyme activity measured with DNP-peptide as substrate

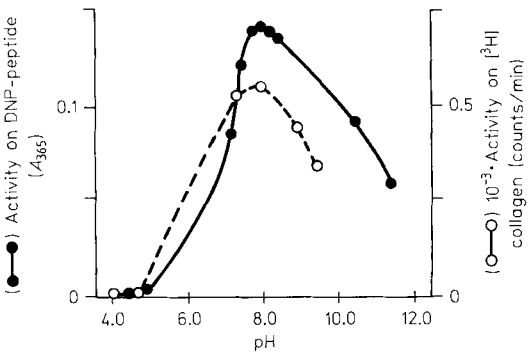

Fig. 4. *pH dependence of Gly-Xaa proteinase activity.* Enzyme preparation, partially purified as indicated in Fig. 3, was incubated in media adjusted to various pH ranges by organic buffers in 30 mM concentrations; the organic buffers used were Hepes, Bes, Tricine, and Tris [32]. The enzyme activity was measured using either denatured [^{3}H]proline-labeled type I collagen or DNP-peptide as substrate. (●——●) Degradation of DNP-peptide measured by absorbance at 365 nm; (○——○) degradation of denatured [^{3}H]collagen measured as Cl_3AcOH-soluble radioactive peptides

DNP-peptide as a substrate. The results indicated that the enzyme activity was readily inhibited by Na_2EDTA (Table 2). No inhibition was noted with α,α′-dipyridyl. $PhMeSO_2F$ in the presence of 10% propanol or 10% Me_2SO (dimethyl sulfoxide) appeared to be inhibitory, but the inhibition was shown to be exclusively due to the solvent in the concentration used (Table 2). MalNEt exhibited a slight stimulation of the enzyme activity. Similarly, a slight activation of the enzyme was noted with OH-HgBzOH (*p*-hydroxymercuribenzoate). Dithiothreitol markedly inhibited the enzyme activity (Table 2). In contrast, iodoacetamide, 6-aminohexanoic acid and soybean trypsin inhibitor did not affect the enzyme activity. Fetal calf serum in 2% concentration had little if any effect on the enzyme activity; in 10% concentration, a slight apparent inhibition was observed, but this was probably due to technical problems with the assay as a result of high protein concentration since no inhibition was noted when denatured collagen was used as substrate (Table 2). Similar results were obtained when denatured type I collagen was used as substrate.

To characterize the substrate specificity of the purified enzyme, type I collagen both in denatured and native forms was subjected to proteolysis; for comparison, the same substrates were incubated with partially purified polymorpho-

Table 2. *The activity of Gly-Xaa proteinase in the presence of various proteinase inhibitors or activators*

Gly-Xaa proteinase, partially purified from human polymorphonuclear leukocytes by concanavalin-A–Sepharose affinity chromatography, gel filtration on Sephacryl S-200, and QAE-Sephadex ion-exchange chromatography, was incubated either with DNP-peptide or denatured [^{3}H]proline-labeled type I collagen, as indicated in Materials and Methods. When DNP-peptide was used as substrate, the activity was expressed as absorbance at 365 nm of the DNP-containing degradation product extracted into an organic solvent; when ^{3}H-labeled collagen was used as substrate, the activity was expressed as counts ^{3}H-labeled radioactive peptides/min rendered Cl_3AcOH-soluble by the enzymatic digestion. The values in parentheses are percentages of the activity observed in controls incubated without an added test compound

Compound tested	Concentration	Activity with DNP-substrate		Activity with denatured collagen	
	mM	A_{365}	(%)	counts/min	(%)
None	–	0.192	(100)	8800	(100)
Na_2EDTA	20.0	0.000	(0)	100	(1)
α,α′-Dipyridyl	10.0	0.197	(103)		
$PhMeSO_2F$:					
in 1% propanol	3.0	0.195	(102)	7400	(87)
in 10% propanol	3.0	0.077	(40)		
10% propanol alone		0.075	(39)	1800	(20)
in 10% Me_2SO	3.0	0.089	(46)		
10% Me_2SO alone		0.094	(49)		
MalNEt	0.2	0.226	(118)		
	1.0	0.242	(126)		
	5.0	0.279	(145)		
	25.0	0.259	(135)		
OH-HgBzOH	1.0	0.272	(142)		
Dithiothreitol	0.04	0.184	(96)		
	0.5	0.081	(42)		
	2.5	0.009	(5)		
Iodoacetamide	10.0	0.220	(115)		
6-Aminohexanoic acid	50.0	0.202	(105)		
Soybean trypsin inhibitor	0.05	0.205	(107)		
Fetal calf serum	(2%)	0.178	(93)	7600	(86)
	(10%)	0.133	(69)	8000	(91)

nuclear leukocyte collagenase and bacterial collagenase (Table 3). The results indicated that about 62% of the denatured collagen was converted to Cl_3AcOH-soluble peptides when incubated with the enzyme isolated by the procedure described above. For comparison, 99% of the radioactively labeled denatured collagen was degraded by baterial collagenase, while very little, if any, proteolysis was noted with polymorphonuclear leukocyte collagenase. In contrast, native type I collagen was almost completely resistant to degradation by the enzyme purified here, while leukocyte collagenase degraded 78% of the same substrate (Table 3).

Table 3. *Degradation of native and denatured type I collagen by Gly-Xaa-proteinase and collagenase isolated from human polymorphonuclear leukocytes*

[^{3}H]Proline-labeled type I collagen, specific activity 3.9×10^7 counts min^{-1} mg^{-1}, was used as substrate; the substrate was denatured by heating at 100 °C for 10 min; the incubations were performed as described in Materials and Methods. Degradation of the substrate was assayed by Cl_3AcOH-precipitation of the ^{3}H-labeled substrate and counting the radioactivity collected on glass-fiber filters; if native collagen was used as substrate, the digestion by the enzymes indicated was followed by brief trypsin and α-chymotrypsin proteolysis, as described in Materials and Methods, and published previously [20]. The values of radioactivity reflect the uncleaved [^{3}H]substrate which was precipitated by Cl_3AcOH; the values in parentheses indicate the relative fraction lost as Cl_3AcOH-soluble [^{3}H]peptides, as compared to a control sample with no enzyme present

Substrate	Enzyme	Degradation	
		counts/min	(%)
Denatured	none	1610	(0)
	Gly-Xaa proteinase	610	(62)
	leukocyte collagenase	1580	(2)
	bacterial collagenase	20	(99)
Native	none	1490	(0)
	Gly-Xaa proteinase	1460	(2)
	leukocyte collagenase	330	(78)
	bacterial collagenase	10	(99)

The time dependence of the degradation was examined by incubating denatured type I collagen with the purified enzyme for time periods varying over 10–360 min; the degradation products were then examined by SDS/polyacrylamide slab gel electrophoresis (Fig. 5). A distinct peptide map was noted in all these samples, and a shift from larger molecular weight fragments to smaller peptides occurred with longer incubation times.

In further studies, the specificity of the enzyme was tested by using unlabeled type I, II, III, IV and V collagens as substrates. Incubation of the enzyme with native collagens revealed no collagenolytic activity, as monitored by SDS/polyacrylamide gel electrophoresis. However, the same collagen substrates, after denaturation by heating at 100 °C, were readily cleaved to small peptides.

The apparent K_m value of the purified enzyme was determined using denatured type I collagen as substrate (Fig. 6). Analyses of the data by double-reciprocal plot indicated an apparent K_m value of 8.2 mg/l (Fig. 6); if the molecular weight of an α1(I) chain, 95000, is used as a basis for calculation, the corresponding molar concentration is 0.086 μM. For comparison, similar measurements of apparent K_m value were also performed with trypsin, an enzyme which rapidly and efficiently degrades denatured collagen [25, 26]. These analyses yielded an apparent K_m value of 0.28 μM, indicating that the K_m of the purified enzyme was approximately three times lower than that for trypsin.

Characterization of the Cleavage Sequence in DNP-peptide

To determine the amino acid sequence at the cleavage site in DNP-peptide, the degradation products, following digestion with the purified enzyme in a 1:50 enzyme:substrate

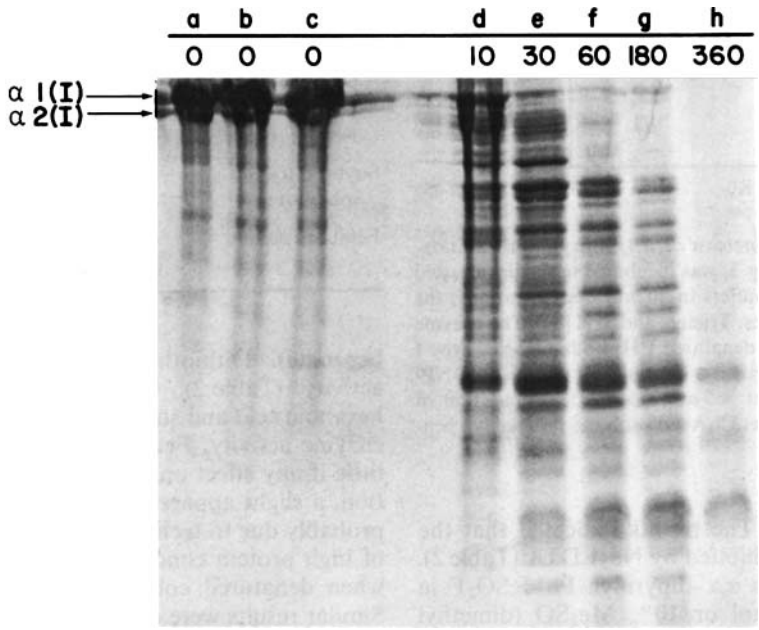

Fig. 5. *Degradation of denatured type I collagen by Gly-Xaa proteinase.* Type I collagen, isolated from human placenta by pepsin solubilization and differential salt precipitation, was incubated with Gly-Xaa proteinase, partially purified as indicated in Fig. 3. After varying incubation periods, the reactions were terminated by the addition of Na_2EDTA, and the digests were examined by SDS/polyacrylamide slab gel electrophoresis using 12% gels. Slot (a) native collagen without enzyme; (b) denatured collagen without enzyme; (c) denatured collagen with enzyme added but not incubated; (d–h) denatured collagen incubated with enzyme for the time periods indicated in the figure (min)

ratio, were analyzed by silica gel thin-layer chromatography. Direct visualization of the gels revealed a degradation product containing a dinitrophenyl group and thus, representing the amino-terminal fragment of the DNP-peptide. This fragment had an R_F of 0.12, while the uncleaved DNP-peptide substrate had an R_F of 0.03. In addition to the peptides containing a dinitrophenyl group, another peptide with an R_F of 0.16 could be visualized by ninhydrin. No other peptides or amino acids could be visualized with ninhydrin, and therefore, this peptide appears to represent the carboxy-terminal cleavage fragment of the DNP-peptide. The degradation products were also partitioned between aqueous and organic phases [18]. Amino acid analysis, after acid hydrolysis, indicated that the amino-terminal fragment, which, unlike the carboxy-terminal fragment or the uncleaved DNP-peptide, was extractable with organic solvent, consisted of proline, glutamic acid and glycine in equimolar concentrations, the ratio of these amino acids being 0.9:1.0:1.0. Thus, the cleavage by the enzyme must have occurred at the Gly-Ile sequence of the DNP-peptide. However, the other sequences, including a Gly-Gln bond, were not cleaved.

Cleavage Sequences in Denatured Type I Collagen

To examine the peptide bonds susceptible to cleavage by the purified proteinase, denatured type I collagen was incubated with the enzyme and the newly created amino termini were dansylated. The dansylated amino acids were then released by acid hydrolysis and separated by polyamide thin-layer chromatography. After a 60-min incubation period, several dansylated amino acids could be detected (Fig. 7).

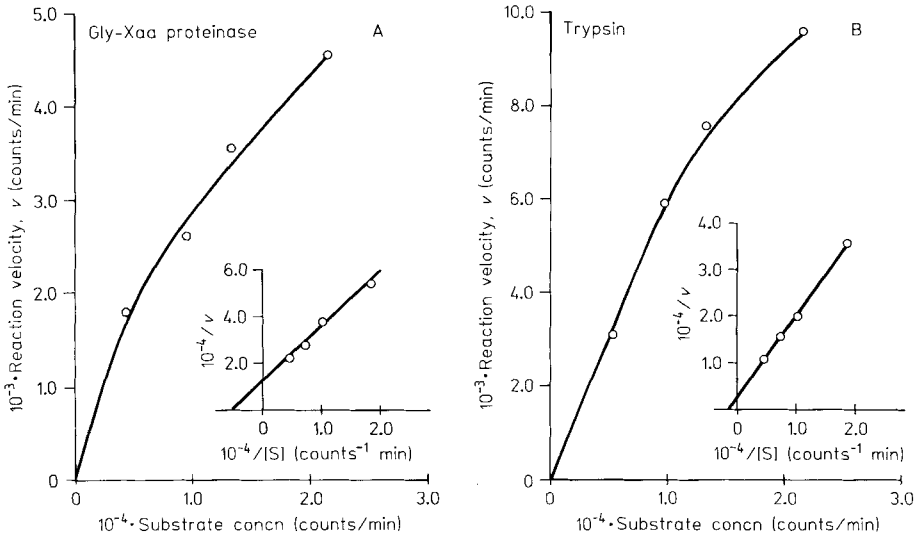

Fig. 6. *Determination of an apparent* K_m *for the Gly-Xaa proteinase using denatured type I collagen as substrate.* (A) [^{3}H]Proline-labeled type I collagen, specific activity 3.9×10^7 counts min^{-1} mg^{-1}, was incubated with highly purified Gly-Xaa proteinase, and the degradation was monitored as Cl_3AcOH-soluble, radioactive peptides. (B) For comparison, similar incubations were performed with trypsin

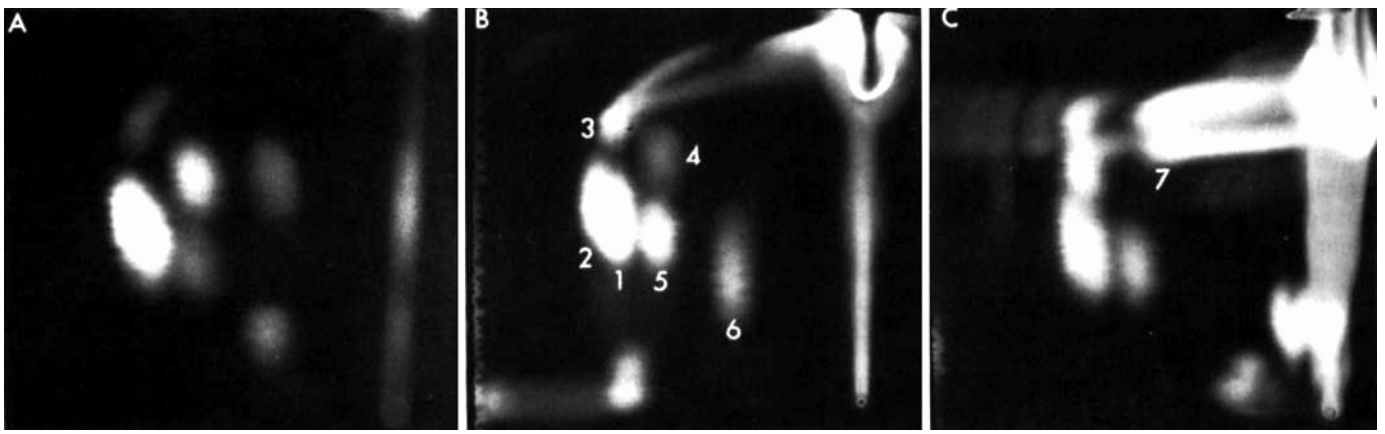

Fig. 7. *Dansyl-monitored end-group analysis of the Gly-Xaa proteinase cleavage fragments derived from denatured type I collagen.* Collagen substrate was incubated with highly purified enzyme for 180 min; in some samples this digestion was followed by a 60-min incubation with carboxypeptidase A. The samples were dansylated and the dansylated amino acids, after acid hydrolysis of the protein, were separated by polyamide thin layer chromatography, as indicated in the text. (A) A mixture of standard dansylated amino acids. (B) Substrate incubated with Gly-Xaa proteinase, but not with carboxypeptidase A. Comparison with standards allows identification of Dns-Leu (1), Dns-Ile (2), Dns-Val (3), Dns-Met (4), Dns-Phe (5) and Dns_2-Lys (6). (C) Substrate incubated first with Gly-Xaa proteinase and then with carboxypeptidase A. Comparison with standards indicates the presence of Dns-Gly (7), in addition to the amino acids identified in B. B and C are from separate experiments and, therefore, the relative quantities of various amino acids are slightly different

Comparison with standard dansylated amino acids allowed identification of Dns-Leu, Dns-Ile, Dns-Val, Dns-Phe, Dns_2-Lys and Dns-Met (Fig. 7). One unidentified dansylated amino acid or dipeptide was also present.

To identify the newly created carboxy-terminal amino acids, the mixture, after enzyme incubation, was further subjected to digestion by carboxypeptidase A. The samples were then dansylated, and the products were separated by polyamide thin-layer chromatography, as above. Comparison between the samples with and without carboxypeptidase A digestion revealed the presence of Dns-Gly in the samples incubated with carboxypeptidase A (Fig. 7). Thus, the proteinase purified in this study cleaves denatures type I collagen at Gly-Xaa sequences, in which Xaa can be a variety of amino acids.

DISCUSSION

In this study, we have demonstrated that human polymorphonuclear leukocytes contain two separate proteinases, one cleaving native and the other one degrading denatured collagen in an apparently specific manner. These two enzymes could be readily separated by concanavalin-A – Sepharose affinity chromatography. The enzyme cleaving denatured collagen was extensively purified and characterized. The enzyme was inhibited by Na_2EDTA, but not by serine proteinase inhibitors. Incubation with MalNEt or OH-HgBzOH, two sulfhydryl reagents, slightly increased the enzyme activity in a manner similar to that which has been demonstrated with polymorphonuclear leukocyte collagenase [30]. Incubation with dithiothreitol inhibited the enzyme activity, suggesting that disulfide bonds are required to maintain the enzyme in an active conformation. Iodoacetamide, 6-aminohexanoic acid, and soybean trypsin inhibitor were not inhibitory. Fetal calf serum in 2% or 10% concentrations did not significantly inhibit the enzyme activity. The active enzyme had a molecular weight of about 105000. The pH optimum of the enzyme was approximately pH 7.8. Thus, the enzyme is a neutral proteinase which requires calcium or another divalent cation for its activity.

The enzyme reported in this study is similar to a proteinase recently purified from human skin explant cultures [15]. The enzyme reported in the latter studies was similarly inhibited by chelating agents, but not by serine proteinase inhibitors nor by sulfhydryl reagents. A difference between the two enzymes was noted, however, in their susceptibility to serum inhibition: the enzyme purified from polymorphonuclear leukocytes was not significantly inhibited by fetal calf serum, while the activity of the enzyme purified from human skin explant cultures was almost completely inhibited by 2.5% serum. This difference may reflect the origin of the purified enzymes, and may have relevance to the site of enzymic action in tissues.

The activity of an enzyme which degrades denatured collagen has previously been reported in preparations of polymorphonuclear leukocytes [16, 17]. In one case, it was suggested that the partially purified enzyme degraded triple-helical TC^A fragments derived from collagen by digestion with collagenase [17]. In our study, such degradation could not be observed. The apparent discrepancy between these results may be explained by the facts that in our incubations MalNEt was included, and that in the previous study the enzyme was not extensively purified.

The enzyme purified in the present study degraded both denatured collagen and a synthetic DNP-peptide. Examination of the degradation products of DNP-peptide by thin-layer chromatography and amino acid analyses indicated that the substrate was cleaved into two fragments and that the cleavage occurred in a Gly-Ile sequence. Analyses of degradation fragments, derived from denatured type I collagen, employing dansyl-monitored end-group analysis, indicated that the major amino-terminal amino acids in degradation fragments were isoleucine and leucine, but lysine, phenylalanine, valine, and methionine were also present. Incubation of the reaction mixture with carboxypeptidase A resulted in the appearance of free glycine. It seems, therefore, that the bond specificity of the purified enzyme is Gly-Xaa in which Xaa can be a variety of amino acids.

Previously, some of the samples degrading denatured collagen have been called 'gelatinases' [6]. This term, however, may not be entirely appropriate in the same sense as the term 'collagenase' is used, since denatured collagen, or gelatin, is readily degraded by a variety of proteolytic enzymes, including trypsin, chymotrypsin, and pepsin [20, 25, 26]. Furthermore, it has been recently demonstrated that human skin fibroblast collagenase, highly purified from cell culture media, is able to cleave denatured collagen [31]. Nevertheless, the 'gelatinases' may show a certain degree of specificity towards denatured collagen. In our study, the relative K_m of denatured type I collagen was about three-times lower for the Gly-Xaa proteinase purified here, as compared with trypsin, an enzyme which rapidly and efficiently degrades denatured collagen. Also, in a previous study, the human skin 'gelatinase' was shown to degrade denatured collagen at a considerably higher rate than other proteins containing susceptible peptide sequences [15]. Since the enzymes degrading denatured collagen have been shown to cleave sequences which have glycine on the carboxyl side and a variety of amino acids on the amino side of the cleavage, we prefer to call these enzymes Gly-Xaa proteinases.

In tissue, the degradation of fibers consisting of triple-helical collagen molecules is initiated most likely only by specific collagenases. After the collagenase digestion, the triple-helical conformation of the cleavage fragments unfolds, and the polypeptides become susceptible to degradation by other proteinases. The Gly-Xaa proteinases probably participate in degradation of the collagenous polypeptides into smaller peptides, and an enzyme, such as that present in human skin [15], may be involved in the normal turnover of collagen in tissues. On the other hand, the Gly-Xaa proteinase present in polymorphonuclear leukocytes, as described in the study, may play an important role in inflammatory disease processes characterized by polymorphonuclear leukocyte infiltration.

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