Reuben, R. C., & Gefter, M. L. (1974) *J. Biol. Chem. 249*, 3843–3850.
Romano, L. J., & Richardson, C. C. (1979) *J. Biol. Chem. 254*, 10476–10482.
Sadowski, P. (1971) *J. Biol. Chem. 246*, 209–216.
Sadowski, P. D. (1977) *Virology 78*, 192–202.
Sadowski, P. D., & Kerr, C. (1970) *J. Virol. 6*, 149–155.
Sadowski, P., McGeer, A., & Becker, A. (1974) *Can. J. Biochem. 52*, 525–535.
Sadowski, P. D., Bradley, W., Lee, D., & Roberts, L. (1980) in *Mechanistic Studies of DNA Replication and Genetic Recombination* (Alberts, B., Ed.) pp 941–952, Academic Press, New York.
Scherzinger, E., Litfin, F., & Jost, E. (1973) *Mol. Gen. Genet. 123*, 247–262.
Studier, F. W. (1972) *Science (Washington, D.C.) 176*, 367–376.
Weinstock, G. M., McEntee, K., & Lehman, I. R. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 126–130.
Yajko, D. M., Valentine, M. C., & Weiss, B. (1974) *J. Mol. Biol. 85*, 323–343.
Yamagishi, M., Fujisawa, H., & Minagawa, T. (1981) *Virology 109*, 148–156.

Purification of Human Neutrophil Collagenase and Production of a Monospecific Antiserum†

Paul Christner,* Denise Damato, Mike Reinhart, and William Abrams

ABSTRACT: Although there is good evidence for the presence of human neutrophil (PMN) collagenase, only moderate purification has been reported. The probable explanation for this fact is that most assays used to specifically measure collagenase activity are not reliable if high levels of several different proteases are also present in the assay mixture. The PMN granule is just such a concentrated mixture. Therefore, polyacrylamide gel electrophoresis was used to identify and quantitate the α1 3/4 and α2 3/4 cleavage products diagnostic for mammalian collagenase. White cells (85% PMN's) were lysed in 0.34 M sucrose and the granules were obtained. The granules were lysed by sonication, and the lysate was chromatographed on a Sephadex G-200 column followed by a Trasylol–Sepharose 4B column. This procedure resulted in a 1350-fold purification and a yield of 75 μg of enzyme/unit of blood. The collagenase was inhibited by ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid but not by sulfhydryl or serine protease inhibitors. The preparation was free of elastase, which has been shown to cleave type III collagen into α1 3/4 and α1 1/4 pieces. The p*I* of collagenase was shown to be 4.7 by isoelectric focusing, and the enzyme lost activity below a pH of 6.5 if collagen was absent. Antiserum was produced by 100-μg injections of the purified collagenase into rabbits. Titers were measured by the enzyme-linked immunosorbent assay. For determination of the specificity, collagenase and PMN extract were isoelectrically focused and blotted onto nitrocellulose. The antibody recognized only one band of protein in the PMN extract, which comigrated with the purified collagenase.

Several authors have reported the presence of human PMN[1] collagenase (Lazarus et al., 1968a,b; Turto et al., 1977; Horwitz et al., 1977; Uitto et al., 1979; Macartney & Tschesche, 1980). These enzymes are similar in action to other collagenases isolated from human skin (Stricklin et al., 1977), rheumatoid synovium (Evanson et al.; 1967; Wooley et al., 1975; Harris, 1972), periodontium (Christner, 1980), macrophages (Senior et al., 1972), and platelets (Chesney et al., 1974), as well as collagenase from other vertebrate sources (Barrett, 1979). Each enzyme has been reported to degrade collagen in its native triple-helical conformation into two fragments a 3/4 and 1/4 piece. The exact site of this cleavage has been determined and is between residues 772 and 773, a glycine–isoleucine bond in the α1 chain and a glycine–leucine bond in the α2 chain (Fietzek et al., 1973; Highberger et al., 1975; Gross et al., 1974).

The fate of the 3/4 and 1/4 fragments depends on the temperature in which the collagenase performs its cleavage. At body temperature (37 °C) both the 3/4 and 1/4 fragments are thermally unstable as triple helices and unwind to form separate α-chain fragments. These fragments are susceptible to general proteolytic digestion because it is the triple helix of collagen that confers unique relative resistance to all known neutral general proteases (McCroskery et al., 1973). When collagenase is assayed at a temperature lower than the melting temperature for the 3/4 helix (32 °C) or 1/4 helix (28 °C), then these fragments each remain as a triple helix (Sakai & Gross, 1967). They are not susceptible to general proteases, can be isolated, and are diagnostic for a true collagenase (Turto et al., 1977; Christner, 1980).

Recently, there has been some confusion about what is a true collagenase. There have been reports in the literature that PMN elastase can degrade certain type III (Gadek et al., 1980) and IV (Mainardi et al., 1980) collagens. Adding to the confusion is the fact that if the fibrillar gel dissolution assay

† From the University of Pennsylvania, School of Dental Medicine, Philadelphia, Pennsylvania 19104 (P.C., D.D., and M.R.), and the Albert Einstein Medical Center, Northern Division, Philadelphia, Pennsylvania 19141 (W.A.). *Received April 16, 1982.* This work was supported by U.S. Public Health Service Grants DE02632 and HL-20994.

[1] Abbreviations: DFP, diisopropyl fluorophosphate; PMSF, phenylmethanesulfonyl fluoride; NEM, *N*-ethylmaleimide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PMN, polymorphonuclear (leucocyte); ELISA, enzyme-linked immunosorbent assay; SAPNA, succinyltrialanine *p*-nitroanilide; Temed, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate; ACD, acid-citrated dextrose; PBS, phosphate-buffered saline.

0006-2960/82/0421-6005$01.25/0

method is used, type I collagen will also appear to be degraded by elastase because the terminal telopeptides are degraded and the gel is solubilized (Starkey, 1977). These facts may be partially responsible for the difficulty that has been encountered in purifying human PMN collagenase. Although there are several methods available for detection of collagenase activity, the actual quantitation of the 3/4 α-chain degradation products of collagen (Turto et al., 1977; Christner, 1980) is the most reliable method now available. It is the belief of these authors that the method used to monitor collagenase activity is critical when isolating PMN collagenase. In support of the role of elastase or other general proteases such as cathepsin G in collagen degradation, we have found that, when a concentrated extract of proteolytic enzyme (the PMN granule) is used as an enzyme source, collagen is degraded without the presence of active collagenase and that methods other than the isolation of 3/4 degradation products on gels are unreliable in following PMN collagenase activity.

Experimental Procedures

Preparation of Granulocytes. Human blood was collected from volunteers by venapuncture or obtained from Interstate Blood Bank, Philadelphia, PA, into ACD and used for the preparation of PMN granules. Within 18 h of drawing, the red cells were removed by dextran sedimentation using 3% Dextran T-500 (Sigma, St. Louis, MO) in saline (Bertino et al., 1963). The supernate was removed by aspiration and centerfuged at 400*g* for 15 min, the pellet resuspended in saline, and any remaining red cells were removed by osmotic shock, by the addition of an equal volume of ice-cold H_2O for 30 s followed by $^1/_2$ volume of 0.6 M KCl. The white cells were then centrifuged at 400*g* for 15 min and washed 2 times in saline. Cell counts performed by a hemocytometer at this point showed 5.49×10^9 cells/unit of blood of which 80–85% were PMN's and the remaining 15–20% primarily lymphocytes.

Separation of Granulocytes from Mononuclear Cells. Although not normally done, granulocytes were separated from mononuclear cells for the results shown in Figure 3. White cells recovered from dextran sedimentations were resuspended in PBS and layered onto a lymphocyte separation medium (LSM solution, Litton Bionetics, Kensington, MD). The procedure supplied by Litton Bionetics with the LSM solution was followed and yielded a granulocyte preparation 97% pure and a lymphocyte preparation 99% pure. The granulocyte preparation contained 97% PMN's, 2.5% lymphocytes, and 0.5% eosinophils and basophils combined. Although monocytes have no granules the monocyte preparation was further treated as described below for comparative purposes.

PMN Granule Extraction. Granules were extracted from the PMN's by lysing the cells in 0.34 M sucrose containing 0.05 M NaCl. Nuclei and unbroken cells were removed by centrifugation at 1000*g* for 10 min. The granules were pelleted at 27000g for 20 min in a SS-34 Sorvall rotor. The supernate was called the high-speed supernate. The granules were resuspended in 4 mL of 0.2 M NaOAc, pH 4.5. Granules were often stored at −20 °C for several weeks before being utilized with no loss in activity. After the granules were thawed, the buffer was immediately adjusted to pH 7.0 with 2 M Tris base. The granules were lysed at 0 °C by sonication with 30-s pulses. The collagenase activity was solubilized by this treatment and separated from the particulate matter by centrifugation at 27000*g* for 10 min. The pellet was resuspended in saline and reextracted 2 times as above. There was essentially no enzyme left in the particulate fraction after three extracting regimens. This fraction was called the granule extract.

Sephadex G-200 Column Chromatography. The granule extracts from 10 units of blood (300–670 A_{280} units) in 10 mL were applied to a Sephadex G-200 column (1.4 m by 1.8 cm) that was equilibrated with 0.05 M Tris(pH 7.5)–0.4 M NaCl–0.001 M $CaCl_2$, and 75 fractions of 5 mL were collected.

Trasylol–Sepharose 4B Column Chromatography. Trasylol (Bayer Werk, Eberfeld, West Germany) was coupled to Sepharose 4B by the Cyanogen bromide activation procedure (Cuatrecasas, 1970). A total of 300 mg of Trasylol to 100 mL of Sepharose 4B was used, which was enough to make a column 22 cm by 1.2 cm. Active fractions from the G-200 column were combined, dialyzed vs. 0.1 mM CaCl, and lyophilized. A 10-fold concentrated sample was applied to the column in 0.01 M Tris, pH 8.0, buffer and collagenase eluted with a 100-mL gradient from 0.01 M Tris, pH 8.0, to 0.05 M Tris, pH 8.0, and 0.4 M NaCl. Fractions of 2.5 mL were collected.

Isoelectric Focusing. Polyacrylamide gels were used as the support for the pH gradient of pH 3.5–10. The electrofocusing range was obtained with an Ampholine mixture (LKB, Uppsala, Sweden) consisting of 2.8 mL, pH 3–10, 0.4 mL, pH 9–11, 0.2 mL, pH 4–6, and 0.2 mL, pH 5–7. Gels were made by mixing 0.47 g of glycerol, 6.33 mL of H_2O, 2.39 mL of 20% acrylamide–8% bis(acrylamide) solution, 476 μL of Ampholine mixture, 30 μL of Temed, and 2 drops of 10% ammonium persulfate. Gels were poured in 1-mL plastic syringes or into an 8 cm square, 0.5 mm thick slab. Gels were prefocused for 1–2 h at 300 V, 4 °C, in a Buchler focusing apparatus, and 100 μL of sample was applied to disc gels or 10–20 μL to slab gels at the basic end of the gel. Gels were focused at 400–500 V, 4 °C, from 2 h to overnight. A mixture of ferritin and hemoglobin, 50 μg each, was used to determine when focusing was complete. The pH gradient was measured by slicing the disc gel into 4-mm slices, adding 1 mL of boiled H_2O, and then determining the pH of the extract. Slab gels were read with a surface pH electrode.

Native Blotting. Focused proteins were blotted onto nitrocellulose by the method of Reinhart & Malamud (1982). Following transfer, upper blots were stained and lower blots were used for ELISA. The latter blots were soaked in 1% BSA in 10 mM Tris-HCl buffer, pH 8.15, for 1 h followed by a 2-h incubation in the same solution containing 2% rabbit anti-collagenase antiserum. Following this incubation, blots were extensively washed (six rinses, 5 min each, 250 mL/rinse with 10 mM Tris-HCl–0.15 M NaCl) and were placed into 0.03% goat antirabbit IgG–horseradish peroxidase conjugate in 1% BSA–10 mM Tris-HCl–0.15 M NaCl. After a 1-h incubation, the blot was washed as above and placed into 10 mM Tris-HCl buffer, pH 7.5, containing 25 μg/mL *o*-dianisidine and 0.01% H_2O_2. Bands were allowed to intensify 30–45 min before the blot was washed with H_2O and dried.

Collagenase Assay System. Our assay system is essentially the same as that of Turto et al. (1977). Guinea pig skin collagen was extracted as previously described (Harris, 1972) and then pepsin treated (Lazarus et al., 1968a). Also, embryonic chick calvaria bones were labeled with [^{14}C]proline and the collagen was extracted as previously described (Harris, 1972). The collagens were then mixed to give a specific activity of 10000–20000 cpm/20 μg of collagen and used as the substrate for collagenase. Assays were carried out at 25 °C in 0.1 M Tris-HCl (pH 7.5)–0.15 M NaCl–0.01 M $CaCl_2$ for various times in a total volume of 150 μL. The amount of collagenase added varied from 10–100 μL. The assay was terminated by adding $^1/_4$ volume of 2% $NaDodSO_4$–0.5%

β-mercaptoethanol and heating for 5 min at 100 °C. The samples were then applied to $NaDodSO_4$ gels for elctrophoresis. The percent collagen degraded was calculated by summing the counts in the α1 and α2 peaks and the 3/4 α1 and 3/4 α2 peaks to obtain total counts. The percent degraded was (α1 3/4 + α2 3/4)/total summed counts.

$NaDodSO_4$ Gels. Our gel system consisted of a running and stacking gel. Polyacrylamide (12.5%) running disc gels were used with an acrylamide:bis(acrylamide) ratio of 192:1. To make 20 100-mm gels, the following mixture was used: 7.5 mL of 3 M Tris, pH 9.0; 25 mL of 30% acrylamide; 6 mL of 0.65% bis(acrylamide); 0.6 mL of 20% $NaDodSO_4$; 20.9 mL of H_2O; 0.05 mL of Temed, and 0.75 mL of 10% ammonium persulfate. The running gel was overlaid with a stacking gel of 3% acrylamide with an acrylamide to bis(acrylamide) ratio of 192:1 and poured in 0.1 M Tris (pH 7.0)–0.6% $NaDodSO_4$. Gels were 110 mm long including the stacker and were run with an Ortec 4100 pulsed constant power supply (Oak Ridge, TN) at 900 pulses and 240 V. Bromphenol blue tracker dye was added to the sample mixture and gels were electrophoresed for 2× the time it took the tracker dye to traverse the entire gel (about 4.5 h).

Elastase Assay. Elastase was assayed by using the synthetic subtrate SAPNA according to the method of Bieth et al. (1974). In a total of 1 mL of reaction mixture there was 0.2 mL of sample, 0.16 mmol of Tris-HCl, pH 8.0, and 1.0 mg of SAPNA. The change in absorbance at 410 nm was recorded with time. The assay readily detected 100 ng/mL human neutrophil elastase.

Preparation of Monospecific Antisera to PMN Collagenase. For purposes of producing antiserum, collagenase from the Trasylol column was further purified by isoelectric focusing in disc gels. A total protein load of 500 μg of material focused on disc gels in the pH range of 3.8–5.0. After being focused, the gel was sliced into 2-mm slices and assayed for activity. The activity, although over 90% lost, focused with one band, at a p*I* of 4.7. This collagenase was used to raise an antibody in rabbits, by injection of an emulsion of the gel slices containing active enzyme in 50% Freund's adjuvant directly into rabbits. A measurable titer was observed after three injections of 100 μg of protein. Titers were generally assayed by a standard ELISA technique. However, for determination of the specificity of the antisera, collagenase was isoelectrically focused and blotted onto nitrocellulose paper. The paper was used to perform an ELISA assay in which the substrate *o*-dianisidine (25 μg/mL) for horseradish peroxidase was precipitated as a brown spot on the nitrocellulose paper where antibody is localized.

Preparation of Affinity Purified Antiserum. Collagenase purified through the Trasylol column step was linked to a Sepharose 4B column [0.1 mg of collagenase/mL of Sepharose (Cuatrecasas, 1970)]. One milliliter of antiserum was passed over the column and eluted with 3 M KCNS. The eluate was dialyzed vs. PBS and used as affinity-purified anticollagenase.

Protein Concentrations. Protein concentrations were determined by assuming 1 mg/mL to have an absorbance of 1 optical density unit at 280 nm except for collagen, which was assumed to be 2.5 at 1 mg/mL at 230 nm.

Results

The procedure used to purify granulocyte collagenase through the Trasylol–Sepharose 4B step is given in Table I. Usually 3 units of blood was used to prepare the granule fraction from whole white cell pellets. The granule fraction was isolated by sonic disruption of the white cells followed by differential centrifugation. It can be seen that isolation of the granule extract resulted in a 2.5-fold increase in specific activity but about a one-third loss in total activity. This loss was probably due to the fact that a certain percentage of the granules lysed when the PMN cell membrane was broken. In support of this interpretation, significant collagenase activity was found in the high-speed supernate after spinning down the granules. After lysis the granule extract was immediately added to a Sephadex G-200 column. As shown in Figure 1, the collagenase activity eluted well separated from the bulk of the protein. The specific activity increased after this gel filtration step by a factor of 7.5 (Table I). In addition, the total activity increased by almost 2-fold. This increase in total activity was probably due to the separation of collagenase inhibitor(s) or general proteases on the G-200 column. The assay time for collagenase was relatively long when testing the impure whole PMN extract or granule extracts, and it is probable that other proteases could have destroyed some of the collagenase during the incubations.

Table I: PMN Collagenase Purification

	sp act.[a] (μg h^{-1} OD^{-1})	x-fold purifcn	yield (%)
whole extract	2.6	1	100
granule extract	7.3	3	88
G-200 eluate	510.0	195	165
Trasylol–Sepharose 4B	3525.0	1355	77

[a] Specific activity is given in micrograms of collagen degraded per hour at 25 °C per optical density unit of PMN protein at 280 nm.

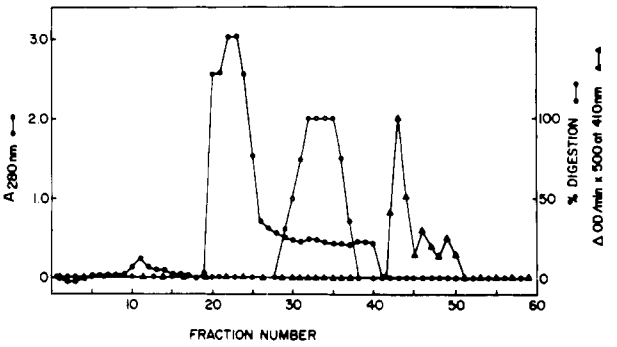

FIGURE 1: Granule extracts were applied to a Sephadex G-200 column and the eluate was monitored for optical density (OD) at 280 nm (●). Collagenase activity, measured as described under Experimental Procedures, is shown by (○). Elastase activity was measured by the SAPNA method described under Experimental Procedures and is shown by (▲).

The active G-200 fractions were pooled and chromatographed on a Trasylol–Sepharose 4B column. The collagenase was eluted by a linear NaCl–Tris-HCl gradient. The results are shown in Figure 2. It can be seen that the collagenase activity binds to the column and elutes at a salt concentration from 0.25 to 0.3 M NaCl. The specific activity of this material was 3525 units (Table I), which represents a 1355-fold purification and a yield of 78%.

Separation of Collagenase and Elastase. Because of the possibility that PMN elastase could be confused with PMN collagenase, several experiments were performed. First, samples from the Sephadex G-200 and Trasylol columns were tested for elastase activity by using the synthetic substrate SAPNA. As can be seen in Figure 1 the elastase and collagenase activities were clearly separated by gel filtration. The peak of elastase activity eluted 15–25 mL later than that of collagenase. The collagenase activity was then applied and eluted from the Trasylol column (Figure 2). The column

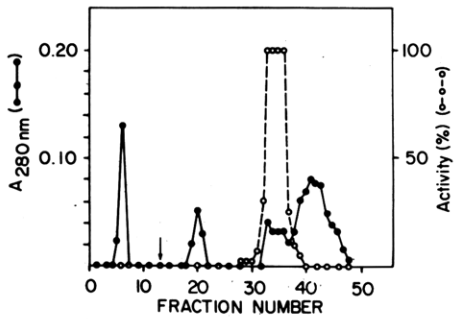

FIGURE 2: Active fractions from a G-200 column eluate were applied to a Trasylol–Sepharose 4B column. A linear gradient of Tris-HCl (pH 8)–NaCl was used to elute the activity. Collagenase activity (O) was measured as described under Experimental Procedures, and the optical density (●) was read at 280 nm. Elastase activity was measured for all fractions as described under Experimental Procedures and was determined to be zero in every fraction.

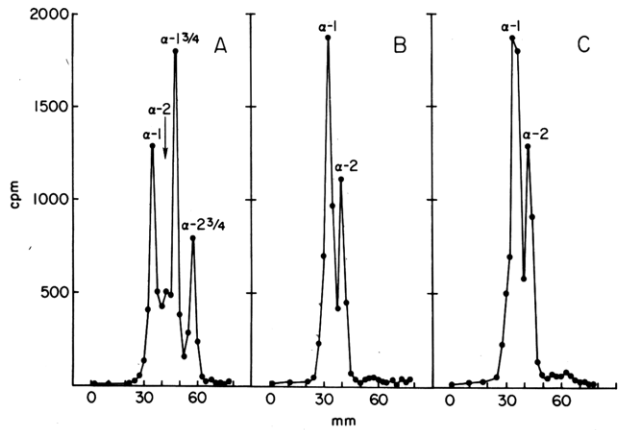

FIGURE 3: 0.2 optical density unit (at 280 nm) of granulocyte high-speed pellet extract (A), monocyte high-speed pellet extract (B), and monocyte high-speed supernate (C) was assayed for collagenase activity for 20 h and electrophoresed on $NaDodSO_4$ gels as described under Experimental Procedures.

fractions were tested for elastase activity; however, no activity could be detected.

Demonstration of Collagenase Activity in Granulocytes. Because of the small amount of collagenase isolated from the 85% pure PMN preparations, a 97% pure PMN preparation was prepared by centrifugation over Ficoll Hypaque. This preparation contained less than 3% monocytes and less than 1% eosinophils and basophils combined. The monocyte preparation consisting primarily of lymphocytes was also obtained from this procedure. Both fractions were tested for collagenase activity (Figure 3); however, only the granulocyte extract showed any collagenase activity.

Characterization of Collagenase. In order to relate PMN collagenase to other human and PMN collagenase we performed several expeeriments to characterize this enzyme.

The purified collagenase was tested to determine its isoelectric point on a slab gel. The p*I* was determined to be 4.7 as seen in Figure 4. To determine if the stained protein band was in fact collagenase, we sliced a duplicate gel (not shown) into 4-mm slices and assayed it for collagenase activity. It was found that most of the activity was lost on isoelectric focusing but that which was recoverable comigrated with the stained band. In a subsequent assay of Trasylol–Sepharose 4B purified collagenase, the enzyme was exposed to a series of pH values from 4 to 7.5 for 1 h at 25 °C in the absence of collagen. It was found that at a pH value below 6.8 there was significant loss of activity. If, however, collagen was present, the enzyme lost little or no activity when exposed to

FIGURE 4: Isolectric focusing of collagenase. The pooled active collagenase fractions from the Trasylol column (Figure 2) were focused on a 0.5-mm slab gel as described under Experimental Procedures (0.01 and 0.05 optical density unit at 280 nm were focused). The collagenase focused with a p*I* of 4.7. Ferritin (p*I* = 4.8) and hemoglobin are shown as standards.

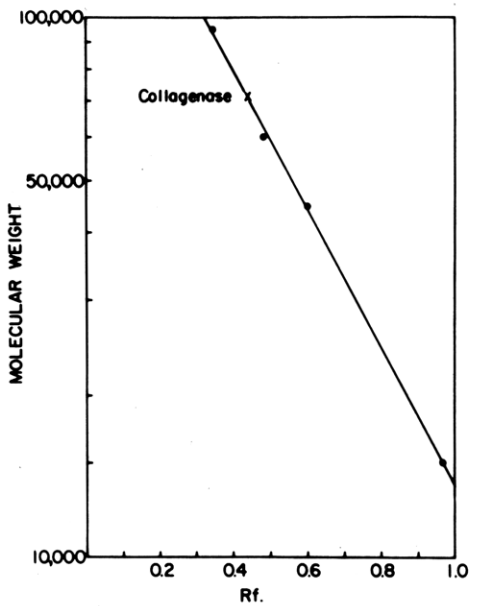

FIGURE 5: Purified collagenase was electrophoresed on $NaDodSO_4$ slab gels. By use of four proteins of known molecular weight, transferrin (95 000), catalase (60 000), ovalbumin (45 000), and hemoglobin (15 000), a standard curve was constructed in which the protein R_f is plotted as a function of the log of its molecular weight. Collagenase as shown had a molecular weight of 70 000.

a pH of 5.5 for 1 h at 25 °C. In addition to determination of the p*I* the molecular weight was also determined in alkaline $NaDodSO_4$ slab gels. Collagenase migrated as a single band with an apparent molecular weight of 70 000 (Figure 5) when compared to standard proteins transferrin (95 000), catalase (60 000), ovalbumin (45 000), and hemoglobin (15 000).

The sensitivity or lack thereof to several different protease inhibitors has been used by several laboratories as a means of characterizing collagenase. Human PMN collagenase has also been characterized in this way by incubation of enzyme and substrate in the presence of 2 mM NEM, 20 mM PMSF, 2 mM DFP, 25 mM EGTA, and 3.5% fresh human serum (Figure 6). From Figure 6 it can be seen that collagenase was active in the presence of each reagent added except 0.025 M EGTA, which completely destroyed the collagenase activity. However, if excess Ca^{2+} was added back to the reaction, the enzyme activity was restored. Human serum did not appear to have any negative effect on the rate of collagen digestion and may have enhanced the rate slightly. However, when the serum was passed over a Sephadex G-200 column and the void volume peak used instead of that of whole serum, a slight inhibition of collagenase activity was noted. In addition to testing the effect of the above reagents, we determined the rate of collagen cleavage by collagenase as a function of pH (Figure 7). It can be seen that the pH optimium was approximately

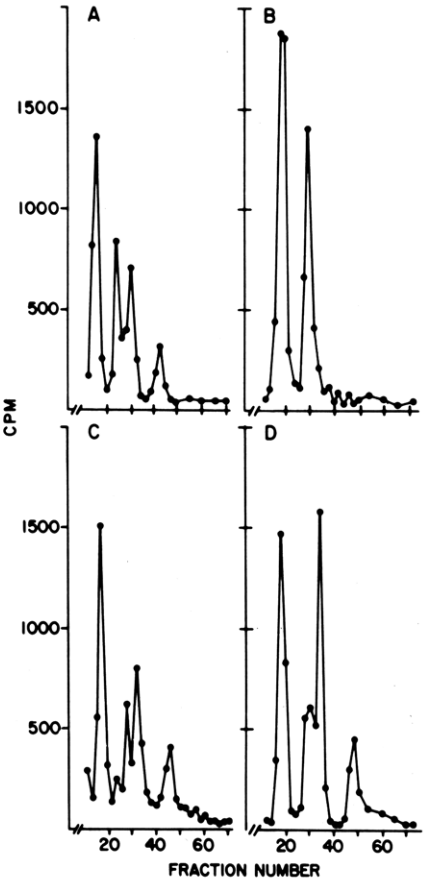

FIGURE 6: Extent of collagenase digestion is shown in the presence of several inhibitors of general proteases. Collagenase activity was measured as described under Experimental Procedures. Inhibitors were added 10 min before collagen to the reaction mixture containing 1 μg of collagenase. In frame A collagenase digestion occurred in the presence of 2 mM NEM–20 mM PMSF–2 mM DFP. In frame B, 25 mM EGTA was also present. In frame C no inhibitors were present. In frame D 50 μL of fresh human serum was present. Frame B was indistinguishable from that of native collagen.

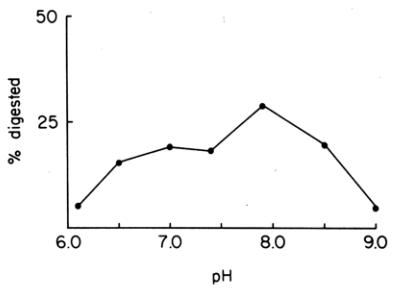

FIGURE 7: Rate of collagenase digestion as a function of pH. Collagenase was assayed as described under Experimental Procedures except that the pH of the reaction mixture was adjusted to the value shown in the figure. After 4-h incubation the reaction was stopped, and the mixture was electrophoresed on $NaDodSO_4$ gels.

8, with an asymmetrically shaped curve that had a prominent shoulder extending from a pH of 6.5 to a pH of 7.5.

Production of a Monospecific Antiserum to Collagenase. Antiserum produced in rabbits was tested for its monospecificity to human PMN collagenase. PMN granule extract and purified collagenase were isoelectrically focused. This procedure resolved the PMN granule proteins into a large number of bands (Figure 8B) and the collagenase into one (Figure 4). The focused protein was then blotted onto nitrocellulose (Reinhart & Mamamud, 1982). When the blotted proteins were exposed to the anticollagenase antiserum, the antiserum localized over the one protein band of purified

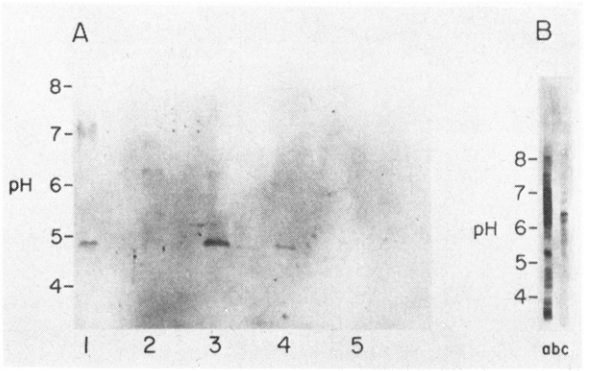

FIGURE 8: (A) Nitrocellulose paper blot of isoelectric focusing gel. 2 μg of purified collagenase from the Trasylol column (lane 3) and 100 μg of the PMN granule extract (lane 4), monocyte high-speed supernate (lane 2), and the monocyte granule extract (lane 5) were isoelectrically focused in a 0.5-mm polyacrylamide slab gel. After being focused, the proteins were blotted onto nitrocellulose paper and then localized with anti-collagenase antiserum followed by goat anti-rabbit serum, conjugated with horseradish peroxidase. The addition of *o*-dianisidine then caused the precipitation of a brown band over any protein localized by the original antiserum. Hemoglobin (lane 1), used as a standard, has intrinsic peroxidase activity and is therefore also stained by this procedure. Ferritin (lane 1), another standard, appears because it is brown. (B) PMN granule extract was isoelectrically focused in tube gels and stained by Coomassie blue. Lane a, 250 μg; lane c, 25 μg; lane b, hemoglobin and ferritin standards.

collagenase (Figure 8A, lane 3) and over the same band in the PMN granule extract (Figure 8A, lane 4).

The monocyte fractions were also tested, and it was found that the antibody gave a slight reaction with the monocyte high-speed supernate fraction (Figure 8A, lane 2). It is not known at present if this reaction is due to the low level of contamination with granulocytes in the monocyte preparation or if the monocytes also contain a small amount of collagenase.

In addition, the antibody was tested for its ability to destroy collagenase activity. Residual collagenase activity was measured after exposure to crude anticollagenase antiserums, affinity-purified antiserum, or appropriate controls. The results (Figure 9) showed that the anticollagenase antibody but not the controls effectively inhibited the collagenase activity in the assay mixture. This evidence indicated we had produced a monospecific antiserum to human PMN collagenase.

Discussion

The importance of collagenase in the remodeling of tissues in response to injury or during growth has been documented by many laboratories. The role of PMN collagenase in the inflammatory response, however, is still not well understood. It can been hypothesized that because PMN's migrate into regions of inflammation that PMN collagenase may be an important enzyme in this process. It has been reported that human PMN collagenase degrades type I collagen 15 times faster than type III, whereas human fibroblast collagenase or rabbit alveolar macrophage digested the two substrates at about equal rates (Horwitz et al., 1977).

In addition granulocyte elastase has been shown to act as a type III collagenase (Mainardi et al., 1980; Gadek et al., 1980). It is obvious then that the type and amounts of collagenase or elastase present during an inflammatory response could effect the eventual collagen matrix architecture in a tissue.

Unfortunately, the differences between human PMN, macrophage, and fibroblast collagenases have not been extensively documented. Wooley et al. (1975) have a report that human fibroblast collagenase was immunologically different from human PMN and macrophage collagenase, but because of the

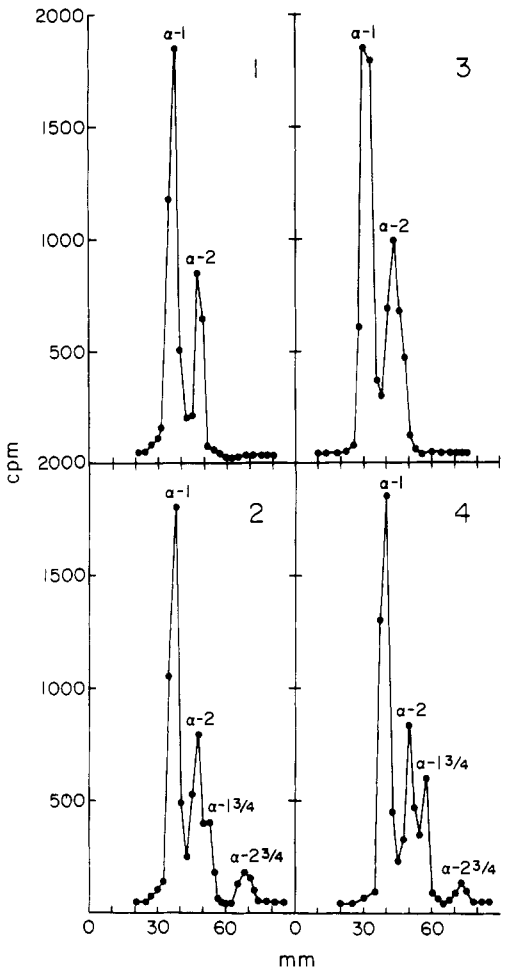

FIGURE 9: (1) 10 μL of rabbit anti-collagenase (35 OD/mL), (2) 10 μL of normal rabbit serum (35 OD/mL), (3) 10 μL of affinity-purified anti-collagenase antiserum (2.7 OD/mL) or (4) 10 μL of affinity-purified anti-enamel antiserum (2.7 OD/mL) were mixed with collagenase for 60 min. Collagen was then added and the mixture incubated for 10 h. *Staphylococcus* A was added and the pellet removed by centrifugation. The supernates were $NaDodSO_4$ treated and electrophoresed on gels.

difficulty in purifying human neutrophil collagenase, supporting biochemical characterization has been lacking.

In this paper we report the first extensive purification of human PMN collagenase. The protein has been purified over 1300 times and has been shown to be a single polypeptide chain on $NaDodSO_4$ gels migrating with molecular weight of about 70000. The protein has been shown to be a calcium-requiring metalloprotease that is not inhibited by the serine protease inhibitors PMSF or DFP or the thiol protease inhibitor NEM. The enzyme was also not inhibited by serum α1-proteinase inhibitor but was partially inhibited by α2-macroglobulin after partial inhibitor purification. These data agree with those reported by Lazarus et al. (1968a) and Uitto et al. (1979) for impure and partially pure enzyme preparations, respectively.

Because elastase has been reported to be a type III collagenase that is caapable of dissolving type I collagen gels, we have taken extreme caution in our purification of neutrophil collagenase. We have used the system described by Christner (1980) and by Turto et al. (1977) for monitoring collagenase activity. When dealing with the PMN granule, it was found that all published methods other than the actual visualization and quantitation of the 3/4 α1 and 3/4 α2 degradation products on $NaDodSO_4$ gels were inadequate to follow collagenase activity because of the high endogenous concentrations of general proteases that cause other assay systems to be nonspecific. For that reason this rather arduous method was used for all assays. In addition, elastase activity was carefully monitored to ensure that it had been removed from the collagenase activity. As can be seen from Figure 2, elastase, a smaller molecule than collagenase, was readily separated by gel filtration chromatography and was subsequently completely absent in thhe Trasylol column eluate.

The amount of collagenase recovered from human PMN's is rather small compared to the amount reported for PMN elastase (Taylor & Crawford, 1975). We recovered only 75–100 μg of collagenase/unit of blood or approximately 10% that reported for elastase from neutrophils. This small recovery may explain in part the difficulty encountered in purifying the enzyme. Because of this small recovery, it could be argued that the collagenase was being isolated from a contaminating white cell. Lymphocytes are the most numerous contaminant and have been reported to contain a collagenase (Simpson et al., 1980). However, upon separation of the granulocytes from the monocytes (primarily lymphocytes) activity was found only in the granulocyte cell population (Figure 3). Corroborating this data is the fact that the antiserum produced to the collagenase recognizes a protein from the granulocyte extract (Figure 9). It seems most likely that the collagenase reported here is from PMN's since the granulocyte population is contaminated with less than 1% other granulocytes: eosinophils and basophils.

From published data it is known that the size of the fibroblast enzyme is about half the size of the PMN enzyme (Bauer et al., 1975; Shinkai & Nagai, 1976). The pH optima of each enzyme are similar (Eisen et al., 1968). The rates of attack by the two enzymes on different types of collagen have been reported to be quite different, but the PMN enzyme used was not pure (Horwitz et al., 1977). This extensive purification of human PMN collagenase will now allow an extensive characterization and comparison with human fibroblast collagenase. Experiments are now in progress to purify fibroblast collagenase and to determine in what ways these two collagenases differ.

References

Barrett, A. J. (1979) *Proteinases in Mammalian Cells & Tissues*, Vol. 2, pp 256, North-Holland Publishing Co., New York.

Bauer, E. A., Stricklin, G. P., Jeffrey, J. J., & Eisen A. Z. (1975) *Biochem. Biophys. Res. Commun. 64*, 232–239.

Bertino, J., Silber, R., Freeman, M., Alentz, A., Achrecht, M., Gabrior, B., & Juennekins, F. (1963) *J. Clin. Invest. 42*, 1899–1904.

Bieth, J., Spiess, E. B., & Wermuth, C. G. (1974) *Biochem. Med. 2*, 350–357.

Chesney, C. M., Harper, E., & Colman, R. W. (1974) *J. Clin. Invest. 53*, 1647–1654.

Christner, P. (1980) *J. Periodontol. 51*, 455–461.

Cuatrecasas, P. (1970) *J. Biol. Chem. 245*, 3060–3065.

Evanson, J. M., Jeffrey, J. J., & Krane, S. (1967) *Science (Washington, D.C.) 158*, 499–502.

Fietzek, P. P., Rexrodt, F. W., Hopper, K. E., & Kühn, K. (1973) *Eur. J. Biochem. 38*, 396–400.

Gadek, J. E., Fells, G. A., Wright, D. G., & Crystal, R. G. (1980) *Biochem. Biophys. Res. Commun. 95*, 1815–1822.

Gross, J., Harper, E., Harris, E. D., Jr., McCroskry, P. A., Highberger, J. H., Corbott, C., & Kang, A. H. (1974) *Biochem. Biophys. Res. Commun. 48*, 1147–1152.

Harris, E. D., Jr. (1972) *J. Clin. Invest. 51*, 2973–2976.

Highberger, J. H., Corbett, C., Kang, A. H., & Gross, J. (1975) *Biochemistry 14*, 2872–2881.

Horwitz, A. L., Hance, A. J., & Crystal, R. G. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 897–901.
Lazarus, G. S., Brown, R. S., Daniels, J. R., & Fulmer, H. M. (1968a) *Science (Washington, D.C.) 159*, 1483–1485.
Lazarus, G. S., Daniels, J. R., Brown, R. S., Bladen, H. A., & Fulmer, H. M. (1968b) *J. Clin. Invest. 47*, 2622–2629.
Macartney, H. W., & Tschesche, H. (1980) *FEBS Lett. 119*, 327–332.
Mainardi, C. L., & Dixit, S. N., & Kang, A. H. (1980) *J. Biol. Chem. 255*, 5435–5441.
McCroskery, P. A., Wood, S., Jr., & Harris, E. D., Jr. (1973) *Science (Washington, D.C.) 182*, 70–71.
Reinhart, M., & Malamud, D. (1982) *Anal. Biochem.* (in press).
Sakai, T., & Gross, J. (1967) *Biochemistry 6*, 518.
Senior, R. M., Bielefeld, D. R., & Jeffrey, J. J. (1972) *Clin. Res. 20*, 88.
Shinkai, H., & Nagai, Y. (1976) *J. Biochem. (Tokyo) 81*, 1261–1268.
Simpson, J. W., Mackler, B. F., O'Neill, P., & Mailman, M. L. (1980) *J. Dent. Res. 59*, 2–10.
Starkey, P. M. (1977) *Protienases in Mammalian Cells and Tissues* (Barrett, A. J., Ed.) pp 57–90, North-Holland Publishing Co., Amsterdam.
Stricklin, G. P., Bauer E. A., Jeffrey, J. J., & Eisen, A. Z. (1977) *Biochemistry 16*, 1607–1615.
Taylor, J. C., & Crawford, I. P. (1975) *Arch. Biochem. Biophys. 169*, 91–101.
Turto, H., Lindy, S., Uitto, V., Wegelius, O., & Uitto J. (1977) *Anal. Biochem. 83*, 557–569.
Uitto, V., Turto, Hi, Huttunen A., Lindy, S., & Uitto, J. (1979) *Biochim. Biophys. Acta 613*, 168–177.
Wooley, D. E., Glanville, R. W., Crossley, M. J., Evanson, J. M. (1975) *Eur. J. Biochem. 54*, 611–622.

Vitamin K Dependent in Vitro Production of Prothrombin†

J. C. Swanson and J. W. Suttie*

ABSTRACT: During prothrombin biosynthesis, glutamyl residues in prothrombin precursor proteins are carboxylated to γ-carboxyglutamyl residues by a vitamin K dependent carboxylase. Calcium-dependent and calcium-independent rat prothrombin antibody subpopulations have been produced and utilized to study the liver microsomal precursors of prothrombin that accumulate when vitamin K action is blocked. A substantial portion of the precursor pool accumulating in the vitamin K deficient or warfarin-treated rat will react with a Ca^{2+}-dependent antibody at high calcium concentration and appears to be partially carboxylated. During in vitro incubation in the presence of vitamin K, the fraction of the precursor pool which is tightly bound to the microsomal membrane appears to be the preferred substrate for the vitamin K dependent carboxylation. A small amount of completely carboxylated rather than a large amount of partially carboxylated products are produced during these incubations. Treatment with a Sepharose-bound prothrombin antibody demonstrated that about 20–25% of the total carboxylated microsomal protein precursor pool consists of prothrombin precursors. This treatment removes an equal amount of total carboxylase activity, and the enzyme is active in this carboxylase precursor–antibody complex.

Vitamin K functions as a cofactor in the posttranslational carboxylation of specific glutamyl residues in microsomal protein precursors to form γ-carboxyglutamyl (Gla) residues in biologically active completed proteins. This modification imparts Ca^{2+}-binding properties to these proteins which, at least in the case of the vitamin K dependent clotting factors, are required for a specific Ca^{2+}-dependent phospholipid association (Suttie, 1978, 1980). Administration of the anticoagulant warfarin, or vitamin K deficiency, causes plasma prothrombin levels to decline and precursor forms to accumulate in the liver (Suttie, 1973). These liver precursors do not appear to bind Ca^{2+}, and, although activation with *Echis carinatus* snake venom (ECV) will generate thrombin from them (Shah et al., 1973), they are not activated under physiological conditions. Prothrombin precursors with p*I*s of 5.8 (Esmon et al., 1975a) and 7.2 (Grant & Suttie, 1976) have been isolated from the liver microsomes of warfarin-treated rats, and additional forms having p*I*s of 5.5, 6.2, and 6.7 have been demonstrated but not yet purified (Graves et al., 1980).

Microsomal preparations obtained from warfarin-treated or vitamin K deficient rats have been shown to produce biological clotting activity (Shah & Suttie, 1974) and to incorporate $^{14}CO_2$ into endogenous protein precursors (Esmon et al., 1975b) in response to the in vitro addition of vitamin K. Following in vitro carboxylation of precursor substrates, $^{14}CO_2$-labeled prothrombin species having p*I*s of both 6.8 (Grant, 1975) and 7.2 (Willingham et al., 1980) have been observed. Due to the number and undefined character of the numerous prothrombin precursor substrates which are carboxylated in vitro, little is known about the fraction of the total precursor pool which is being modified, the nature of the specific precursor(s) acting as a substrate, or the extent to which these precursors are being carboxylated.

Calcium binding induces conformational changes in prothrombin, and a conformationally specific subpopulation of antibodies directed against the γ-carboxyglutamic acid rich region of bovine prothrombin has previously been isolated and characterized (Wallin & Prydz, 1977; Furie et al., 1978; Madar et al., 1980). These antibodies have been used to study metal ion induced conformational changes (Furie et al., 1979; Madar et al., 1982) and plasma distribution of uncarboxylated prothrombin species (Blanchard et al., 1981). In this study, we report the isolation of conformationally specific antibodies directed against rat prothrombin and their use to characterize

† From the Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin—Madison, Madison, Wisconsin 53706. *Received March 30, 1982.* This work was supported by the College of Agricultural and Life Sciences of the University of Wisconsin—Madison and in part by Grant AM-14881 of the National Institutes of Health.

0006-2960/82/0421-6011$01.25/0