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Purification to Homogeneity of Latent and Active 58-Kilodalton Forms of Human Neutrophil Collagenase†

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ABSTRACT: Latent and active 58-kDa forms of human neutrophil collagenase (HNC) have been purified to homogeneity. Buffy coats were extracted in the presence and absence of phenylmethanesulfonyl fluoride to generate crude starting preparations that contained latent and active HNC, respectively. The buffers used in preparing these extracts and for all subsequent chromatographic steps contained NaCl at a concentration of 0.5 M or greater, 0.05% Brij-35, concentrations of $CaCl_2$ of 5 mM or greater, and (when feasible) 50 μM $ZnSO_4$ to stabilize the HNC. The collagenase activity in the buffy coat extracts was adsorbed to a Reactive Red 120-agarose column at pH 7.5 in 0.5 M NaCl and was eluted when the NaCl concentration was increased to 1 M. The active and *p*-(chloromercuri)benzoate-activated latent enzymes were next adsorbed to a Sepharose-CH-Pro-Leu-Gly-NHOH affinity resin in 1 M NaCl at pH 7.5 and desorbed at pH 9 to give a fraction containing only HNC and a small amount of neutrophil gelatinase. The latter enzyme was removed by passage over a gelatin-Sepharose column in 1 M NaCl at pH 7.5. The purified samples of active and latent HNC were obtained with typical cumulative yields of 32 and 82% and specific activities toward soluble rat type I collagen at 30 °C of 7200 and 12000 μg min^{-1} mg^{-1}, respectively. These specific activities are markedly higher than previously reported for HNC. Both active and latent HNC exhibit a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis both in the presence and in the absence of 2-mercaptoethanol. The mobility of latent HNC is consistent with a molecular weight of approximately 58K, with the active form exhibiting a slightly lower (<1-2K) molecular weight.

The breakdown of interstitial collagens in all higher organisms is believed to be initiated by specific collagenases (EC 3.4.24.7) (Birkedal-Hansen, 1987; Van Wart & Mookhtiar, 1990). The key feature of these enzymes is their ability to hydrolyze the triple-helical collagen monomers of collagen fibrils at a specific locus to produce characteristic three-fourths and one-fourth fragments. Because of their involvement in both normal and pathological connective tissue catabolism, it is vital that the collagenases produced by different human cells be isolated and characterized in detail. The most intensively studied human collagenase is that originally isolated from skin fibroblast cultures (Stricklin et al., 1977). Human fibroblast collagenase (HFC)[1] is a metalloproteinase that is produced only by de novo synthesis and secreted into the extracellular space as a zymogen. The sequence of the secreted zymogen, which has a molecular weight of 52K, has been

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[1] Abbreviations: HNC, human neutrophil collagenase; HFC, human fibroblast collagenase; HNG, human neutrophil gelatinase (type IV collagenase); SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Brij-35, polyoxyethylene(23) lauryl ether; PCMB, *p*-(chloromercuri)benzoate; PMSF, phenylmethanesulfonyl fluoride.

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deduced from a cDNA clone (Goldberg et al., 1986). The mechanism of activation of the zymogen (Stricklin et al., 1983; Grant et al., 1987; Springman et al., 1990) has been extensively studied, and the collagen (Welgus et al., 1981) and peptide (Fields et al., 1987) specificities of the active enzyme have been examined.

The collagenases produced by most other human cells, including those from synovial cells (Brinckerhoff et al., 1987), transformed osteoblasts (Otsuka et al., 1984), keratinocytes (Lin et al., 1987), and macrophages (Campbell et al., 1987), are either identical with or closely resemble HFC. The only other distinct human collagenase is that found in neutrophils. In contrast to HFC, human neutrophil collagenase (HNC) is stored within the cell in specific granules (Lazarus et al., 1968; Murphy et al., 1977) and can be released by a variety of stimuli (Oronsky et al., 1973; Cheung et al., 1983; Hibbs et al., 1984; Weiss et al., 1985; Hasty et al., 1986). HNC is immunologically distinct from HFC (Hasty et al., 1984, 1987a) and has been reported to have a different molecular weight (Murphy et al., 1980; Macartney & Tschesche, 1983; Sorsa et al., 1985; Hasty et al., 1986) and different collagen specificity (Horwitz et al., 1977; Hasty et al., 1987b). Unfortunately, a detailed comparison of these two human collagenases has been hampered by the lack of purification procedures that give chromatographically pure HNC.

Although HNC was one of the first mammalian collagenases to be discovered (Lazarus et al., 1968), and numerous methods have been used to purify it (Murphy et al., 1977, 1982; Turto et al., 1977; Engelbrecht et al., 1982; Christner et al., 1982; Macartney & Tschesche, 1983; Williams & Lin, 1984; Sorsa et al., 1985; Callaway et al., 1986; Hasty et al., 1987b; Sorsa, 1987), no procedures currently exist for the preparation in high yield of chromatographically purified latent enzyme that can be activated to high specific activity. The purification of HNC is complicated by the fact that it is unstable and loses activity during chromatography. In addition, latent HNC has a tendency to undergo spontaneous activation as well as autolytic degradation on purification. These problems have contributed significantly to the disagreement concerning the basic properties of this enzyme, including its molecular weight, the basis for its latency, its collagen substrate specificity, and its mechanisms of activation. Hasty and associates (Hasty et al., 1986) have shown that neutrophils secrete two major species of HNC with molecular weights of approximately 58K and 75K. In this study, both active and latent 58-kDa forms of HNC have been purified to homogeneity in high yield with specific activities for the active and *p*-(chloromercuri)benzoate (PCMB)-activated latent species that are markedly higher than reported previously for HNC or any other human collagenase. This purification has helped to clarify some of the disagreements concerning the basic properties of HNC and allowed a more detailed comparison with HFC (Mallya et al., 1990). The purification of the 75-kDa species requires a much different procedure which will be described later (Springman, Dioszegi, and Van Wart, unpublished results).

Materials and Methods

Materials. Buffy coats from fresh or outdated human blood were obtained from the American Red Cross; Brij-35 was from Calbiochem; Sepharose-4B and activated CH-Sepharose were from Pharmacia Fine Chemicals; PM-10 membranes, Centricons, and a dye–ligand test kit were from Amicon Corp.; sodium dodecyl sulfate was from Bio-Rad; bicinchoninic acid was from Pierce; spectrographically pure $ZnSO_4$ was from Johnson Matthey; and Reactive Red 120–agarose, PCMB, benzoyl-Tyr ethyl ester, succinyl-Ala-Ala-Val-*p*-nitroanilide, Leu-*p*-nitroanilide, phenylmethanesulfonyl fluoride (PMSF), and deoxyribonuclease I were from Sigma Chemical Co. Latent HFC was a gift from Dr. Henning Birkedal-Hansen of the University of Alabama at Birmingham.

Synthesis of Affinity Resins. Gelatin was immobilized on Sepharose-4B by the cyanogen bromide activation method (March et al., 1974), and Pro-Leu-Gly-NHOH was immobilized by reaction with activated CH-Sepharose (Moore & Spilburg, 1986).

Enzymatic Assays. Collagenase activity was determined by measuring the initial rate of hydrolysis of soluble ^{3}H-acetylated rat tail tendon type I collagen at 30 °C in 50 mM Tricine, 0.2 M NaCl, and 10 mM $CaCl_2$, pH 7.5, containing 0.05% Brij-35 and 50 μM $ZnSO_4$ at a substrate concentration of 100 μg/mL (Mallya et al., 1986). Assays for the hydrolysis of nonradiolabeled rat tail tendon type I collagen fibrils were carried out in the same buffer at 37 °C as described in the following paper (Mallya et al., 1990). Gelatinase activity was measured in the same buffer using heat-denatured ^{3}H-acetylated rat tail tendon type I collagen at a concentration of 100 μg/mL at 37 °C (Harris & Krane, 1972). Crude and pure samples of latent HNC were activated by incubation with 0.5 and 0.1 mM PCMB, respectively, at 23 °C for 20 min. HFC was activated with trypsin as described in the following paper (Mallya et al., 1990). The samples for all gelatinase assays were pretreated with 1 mM PMSF to inactivate serine proteinases. Thus, most of the gelatinase activity measured was due to human neutrophil gelatinase (HNG). In all cases, 1 mM 1,10-phenanthroline was added to control assays and the absence of activity used to confirm that the gelatinase activity arose from a metalloproteinase. Aminopeptidase activity was measured spectrophotometrically using Leu-*p*-nitroanilide as substrate (Van Wart & Lin, 1981) at 23 °C in 50 mM Tricine, 0.4 M NaCl, and 10 mM $CaCl_2$, pH 7.5. Elastase and cathepsin G were also assayed spectrophotometrically using succinyl-Ala-Ala-Val-*p*-nitroanilide (Engelbrecht et al., 1982) and *N*-benzoyl-Tyr ethyl ester (Gerber et al., 1974) as substrates, respectively, at 23 °C in 50 mM Tricine, 1 M NaCl, and 10 mM $CaCl_2$, pH 7.5.

Protein Determination. The concentration of HNC was determined by the bicinchoninic acid microprocedure (Smith et al., 1985) using HFC as a standard. Solutions of HFC were prepared in buffers that contained identical concentrations of Brij-35, $CaCl_2$, etc. as those in which the pure HNC samples were dissolved. The concentration of HFC was determined spectrophotometrically using $\epsilon_{280} = 6.8 \times 10^4$ M^{-1} cm^{-1} (Birkedal-Hansen et al., 1988).

Chromatography. All chromatographic procedures were performed at 4 °C with controlled flow rates. Fractions indicated by upper case letters were quick-frozen in dry ice/acetone baths.

Gel Electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed as described (Laemmli, 1970). The SDS contained less than 1% of the C_{14} and C_{16} alkyl sulfates. When gels were run under denaturing conditions, the samples were diluted with an equal volume of 0.19 M Tris, pH 6.8, containing 0.15 M EDTA, 8 M urea, 2% SDS, 20% glycerol, 0.05% Bromophenol Blue, and 6% 2-mercaptoethanol, heated in a boiling water bath for 3 min, and cooled to 23 °C before applying them to gels. Gel zymography experiments were carried out at 4 °C with samples that were incubated at 23 °C for 30 min with 2% SDS prior to application to the gels. Collagen (Birkedal-Hansen & Taylor, 1982) and gelatin (Hibbs et al., 1985) zymography

Table I: Purification of Active Human Neutrophil Collagenase

		collagenase		gelatinase		elastase		aminopeptidase		cathepsin G	
fraction	protein (mg)	sp. act. (μg min^{-1} mg^{-1})	yield (%)	sp. act. (μg min^{-1} mg^{-1})	yield (%)	sp. act. (nmol min^{-1} mg^{-1})	yield (%)	sp. act. (nmol min^{-1} mg^{-1})	yield (%)	sp. act. (nmol min^{-1} mg^{-1})	yield (%)
buffy coat extract	4100	1.1	100	38	100	46	100	7.3	100	63	100
Reactive Red											
A	3400	0.56	42	24	52	3.3	6.0	5.6	64	35	47
B	170	14	53	170	18	5.6	0.50	0	0	0	0
C	120	0	0	0	0	40	2.6	0	0	0	0
affinity resin											
A	120	4.7	13	36	2.8	5.9	0.4	0	0	0	0
B	1.3	2200	63	4000	3.0	0	0	0	0	0	0
gelatin–Sepharose											
A	0.20	7200	32	470	<0.1	0	0	0	0	0	0
B	ND[a]	ND	3.8	ND	3.3	0	0	0	0	0	0

[a] ND, not determined.

Table II: Purification of Latent Human Neutrophil Collagenase

		collagenase		gelatinase		elastase		aminopeptidase		cathepsin G	
fraction	protein (mg)	sp. act. (μg min^{-1} mg^{-1})	yield (%)	sp. act. (μg min^{-1} mg^{-1})	yield (%)	sp. act. (nmol min^{-1} mg^{-1})	yield (%)	sp. act. (nmol min^{-1} mg^{-1})	yield (%)	sp. act. (nmol min^{-1} mg^{-1})	yield (%)
buffy coat extract	1800	16	100	13	100	0	0	16	100	0	0
Reactive Red											
A	1300	4.5	20	12	66	0	0	27	120	0	0
B	290	100	100	16	11	0	0	3.6	2.0	0	0
C	120	0	0	24	13	0	0	0	0	0	0
affinity resin											
A	280	2.9	2.8	0	0	0	0	1.1	1.8	0	0
B	3.0	6200	65	730	9.4	0	0	0	0	0	0
gelatin–Sepharose											
A	2.0	12000	83	170	1.4	0	0	0	0	0	0
B	ND[a]	ND	1.7	ND	5.7	0	0	0	0	0	0

[a] ND, not determined.

were performed according to published procedures. Denaturing gels were stained by the silver stain method (Merril et al., 1981), while the collagen films and the gels containing gelatin were stained with Coomassie Blue (0.2% in 50% methanol and 10% acetic acid in water) and destained with 10% methanol/5% acetic acid in water.

Results and Discussion

Preparation of Crude Buffy Coat Extract: Buffy coats from outdated blood are a good, readily available starting material for the purification of HNC. Since the only white cells that contain collagenase are granulocytes (Christner et al., 1982), the buffy coats were extracted directly without further fractionation. The alternative of isolating viable neutrophils from fresh blood and stimulating secretion of HNC with phorbol myristic acetate (Hasty et al., 1986) or other agents (Oronsky et al., 1973; Cheung et al., 1983; Hibbs et al., 1984; Weiss et al., 1985) produces a starting material of higher initial purity but gives a lower yield and is harder to scale up. The purification procedure described below gives the same results whether buffy coats from fresh or outdated blood are used as the starting material. The buffy coats obtained from outdated blood, however, generally contain a larger number of contaminating erythrocytes, and the procedure described below pertains to this more crude starting material.

The buffy coats were diluted with an equal volume of 0.9% NaCl and centrifuged for 10 min at 1000*g* at 4 °C. The middle fractions, which were enriched in neutrophils, were carefully collected and subjected to hypotonic hemolysis for 1 min. The resulting suspension was made isotonic with 3.6% NaCl, and the cells were pelleted by centrifugation for 10 min at 3000*g* at 4 °C. The cells were washed twice with 0.9% NaCl, suspended in an equal volume of 10 mM Tris, 1 M NaCl, and 5 mM $CaCl_2$, pH 7.5, containing 0.05% Brij-35 and 50 μM $ZnSO_4$, and homogenized in a Virtis blender, and the homogenate was diluted to approximately 300 mL with the same buffer. If the latent form of HNC was to be isolated, PMSF (100 mM in 2-propanol) was added to a final concentration of 1 mM. The suspension was then freeze-thawed 7 times in dry ice/acetone and in a 35 °C water bath, respectively. The cell debris was pelleted by centrifugation at 4 °C at 16000*g* for 1 h. The supernatant was diluted with 10 mM Tris/5 mM $CaCl_2$, pH 7.5, containing 0.05% Brij-35 and 50 μM $ZnSO_4$ so that the final NaCl concentration was 0.5 M. A few flakes of solid deoxyribonuclease I were added to the supernatant along with enough $MgSO_4$ so that its final concentration was 0.5 mM. The solution was allowed to stand overnight, after which it was clarified by centrifugation at 16000*g* for 3 h. This supernatant, referred to as the crude buffy coat extract, was the starting material for the chromatographic purification.

The crude buffy coat extract contains large quantities of hemoglobin as well as neutrophil proteins such as myeloperoxidase, elastase, cathepsin G, aminopeptidase, HNG, and HNC. Treatment of the buffy coat suspension with PMSF immediately prior to the freeze–thawing step (see above) effectively prevents activation of latent HNC, presumably by preventing proteolysis by serine proteinases. If this treatment is omitted, the HNC in the crude extract is fully activated. Results are described below for the purification of both active and latent HNC from such preparations (Tables I and II, respectively). The fate of the contaminating proteinases in both of these purifications has been followed, with the exception of the elastase and cathepsin G present in the latent preparations, since they are inactivated by the PMSF treatment.

Reactive Red 120–Agarose Chromatography. A typical buffy coat extract from 30 units of blood contains approxi-

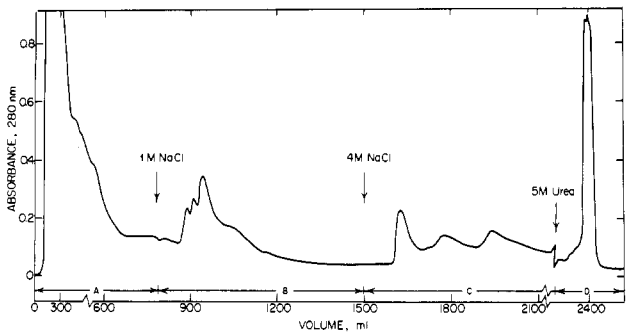

FIGURE 1: Chromatography of crude human buffy coat extract containing active HNC over Reactive Red 120–agarose at 4 °C. The sample was applied to the column and eluted as described in the text.

mately 2–4 g of protein, much of which is hemoglobin. Thus, the first fractionation step was designed to separate the collagenase activity from the bulk of the hemoglobin. A number of crude fractionation steps such as ammonium sulfate and organic solvent precipitation were tested. Such procedures either resulted in low yields of collagenase or failed to separate the collagenase activity efficiently from the hemoglobin. Several types of ion-exchange steps were also unsuccessful for the same reasons (see below). Five dye–ligand test resins were evaluated for their ability to separate the collagenase activity from the hemoglobin. The Reactive Red 120–agarose resin proved to be the most effective and was used as the first step in the purification.

Crude buffy coat extract (250–500 mL) from either a latent or an active preparation dissolved in 10 mM Tris, 0.5 M NaCl, and 5 mM $CaCl_2$, pH 7.5, containing 0.05% Brij-35 and 50 μM $ZnSO_4$ was applied to a Reactive Red 120–agarose column (2.5 × 60 cm) that had been previously equilibrated with the same buffer, except devoid of Brij-35, at a flow rate of 25 mL/h. The column was washed with this buffer until the absorbance reached the base line to give fraction A (Figure 1). The column was then washed with the same buffer containing 1 M NaCl and 0.05% Brij-35 until the absorbance again reached the base line (fraction B). Finally, the tightly bound proteins were eluted with 10 mM Tris, 4 M NaCl, and 5 mM $CaCl_2$, pH 7.5, containing 0.05% Brij-35 (fraction C). The column was regenerated by washing with 3 volumes of 50 mM Tris, 0.2 M NaCl, and 10 mM $CaCl_2$, pH 7.5, containing 5 M urea (fraction D).

The yields of proteolytic activities and total protein in each fraction of the purification are summarized in Tables I and II for an active and a latent collagenase preparation, respectively. In both cases, 60–80% of the protein in the crude extract elutes in Reactive Red 120–agarose fraction A. This fraction contains virtually all of the hemoglobin and aminopeptidase activity, and most of the gelatinase activity. In the case of an active preparation, all of the cathepsin G and most of the elastase activity that is recovered also elutes in fraction A (Table I). Since the yield of elastase is low, it is possible that some enzyme remains tightly bound to the resin and is eluted in the urea wash. Most of the collagenase activity in both preparations is in fraction B together with 10–20% of the gelatinase activity. The actual yield of collagenase in this fraction depends on the amount of contaminating hemoglobin in the crude starting material, where Tables I and II represent the lower (53%) and upper (100%) range of yields encountered, respectively. When there is more hemoglobin present, some collagenase activity elutes in fraction A (e.g., Table I), probably due to competition for binding sites on the resin. When fraction A is rechromatographed on the Reactive Red column, all of the collagenase activity is recovered in fraction B. When the amount of contaminating hemoglobin is small, it is not uncommon to achieve a yield of HNC in this step of greater than 90% (Table II). We have performed this purification step over 15 times with a yield in fraction B that ranged from 53 to 110%. This step increases the specific collagenase activity by approximately 10-fold. Myeloperoxidase, which has a characteristic green color, elutes in fraction C.

Stabilization of HNC. At higher stages of purity, HNC becomes unstable, and all attempts to purify it further are unsuccessful unless there is strict adherence to several procedures. First, solutions of HNC prepared in buffers with low NaCl concentrations lose activity rapidly, probably due to adsorption of the enzyme to glass and plastic surfaces and chromatographic supports (Murphy et al., 1982). Such losses are also experienced during the concentration of protein samples at low NaCl concentrations and are probably a consequence of the low ionic strength of these solutions. For example, the 10-fold concentration of partially purified HNC by ultrafiltration using a PM-10 membrane results in losses of greater than 70% when carried out at a NaCl concentration of 0.05 M. However, when performed at 0.8 M NaCl, over 90% of the activity is recovered. A similar loss in activity is also observed when HNC is dialyzed against low ionic strength buffers, a step that is often needed prior to ion-exchange chromatography. The purification scheme described below was designed to avoid steps that required exposure of HNC to solutions with NaCl concentrations less than 0.5 M.

Second, even in the presence of 0.5 M NaCl, samples of HNC lose activity during later stages of purification without additional stabilization. This could be related to the instability of the enzyme at high dilution. Sugars can stabilize proteins in dilute solutions (Harris & Cartwright, 1977; Lee & Timasheff, 1981). However, 0.3 M sucrose and 0.1 M fructose were found to lower the activity of HNC by 64 and 31%, respectively. The surfactant Brij-35, which has been used to stabilize pig synovial collagenase during purification (Cawston & Tyler, 1979), was found to dramatically increase the chromatographic yields of HNC at a concentration of 0.05%, while having no deleterious effect on HNC or on the collagenase assay used. In fact, in the two chromatographic steps described below, the absence of Brij-35 in the buffers results in almost total loss of activity. One drawback to the use of Brij-35 is that its micelles concentrate along with HNC when PM-10 membranes are used. This makes it impractical to recover HNC from extremely dilute solutions containing Brij-35.

Third, it has been found that the activity of HNC is stabilized by dilute zinc ions. Since HNC is thought to be a zinc metalloproteinase (Mookhtiar et al., 1986), this could prevent a loss of zinc from the active site, but the basis for this effect is not yet firmly established. The optimum concentration to be included in chromatographic buffers has been determined to be 50 μM (Mallya & Van Wart, 1989). Fourth, HNC is more stable to almost all manipulations when kept in the latent form. For example, incubation of partially purified latent and active samples of HNC which were free of elastase and cathepsin G at 4 °C for 24 h results in losses of 0 and 37%, respectively. Similar losses occur on dialysis and concentration. The *amount* of collagenase activity in a crude buffy coat extract is generally much higher for latent than active preparations (Tables I and II), suggesting either that the active enzyme is inherently less stable or that HNC is destroyed by the serine proteinases in the active preparation, as noted by other investigators (Murphy et al., 1980; Sorsa et al., 1985;

Callaway et al., 1986). Furthermore, the yields of collagenase in the chromatographic steps are also generally better for latent HNC. Thus, treatment of the crude extracts with PMSF increases the yield of HNC in the extraction, keeps the enzyme in the latent form, and renders it more stable in subsequent chromatographic steps. Last, freeze–thawing has a deleterious effect on the activity of HNC. The purified enzyme is most stable when quick-frozen in dry ice/acetone and stored at −70 °C.

With the results of these stabilization experiments in mind, a number of published procedures were used in an attempt to purify HNC from Reactive Red 120–agarose fraction B. A variety of ion-exchange steps were screened, but all proved ineffective. Anion-exchange chromatography over DEAE-Sephacel was carried out as described by Macartney and Tschesche (1983) by applying the sample in 0.1 M Tris/50 mM $CaCl_2$, pH 7.4. In our hands, HNC did not bind to the resin in this buffer. Increasing the pH to 8.5 also failed to induce HNC to bind. On decreasing the concentration of Tris to 10 mM and that of $CaCl_2$ to 5 mM at pH 8.5, HNC did not bind; however, NaCl gradient elution gave poor yields and very little purification. Similar experiments carried out with the stronger anion-exchange resin QEAE-Sephadex as described by Turto et al. (1977) or with DEAE-cellulose gave the same poor results. The failure of HNC to bind to DEAE-cellulose under the conditions described is in agreement with the observations of other workers (Murphy et al., 1982; Hasty et al., 1986). However, since HNG is bound tightly by this resin, this is an effective way to remove it from HNC.

Cation-exchange chromatography was also attempted by applying Reactive Red 120–agarose fraction B to CM-cellulose at pH 7.5 in 0.05 M NaCl, but HNC eluted with 30% of the total protein in the starting buffer. Another procedure that was attempted involved the use of collagen–Sepharose as an affinity resin (Macartney & Tschesche, 1983). Even after circulation of partially purified HNC over this column for 24 h under the specified conditions, no binding of HNC to the matrix was observed. A published step (Callaway et al., 1986) that was compatible with the stabilization conditions delineated above was Sephacryl S-200 chromatography in buffer containing 0.5 M NaCl, but this gave only a 2-fold increase in specific activity. Thus, none of these procedures were used in our purification.

Sepharose-CH–Pro-Leu-Gly-NHOH Affinity Chromatography. Attention was turned to an affinity resin developed by Moore and Spilburg (1986), since it has been reported to effectively purify HFC under conditions that allowed the application of the sample in 0.5 M NaCl. The affinity ligand Boc-Pro-Leu-Gly-NHOH was synthesized and tested for inhibition of HNC and HNG in 50 mM Tricine, 0.2 M NaCl, and 10 mM $CaCl_2$, pH 7.5, containing 0.05% Brij-35. The IC_{50} values for the two enzymes were found to be 59 and 74 μM, respectively. The Boc group was removed and the peptide immobilized on activated CH-Sepharose. The resulting resin was found to bind both HNC and HNG when used under the recommended conditions (Moore & Spilburg, 1986). A number of alternate elution conditions involving the presence of PCMB, Brij-35, and variable NaCl concentrations were tested, and the optimum conditions are described below.

Fraction B from the Reactive Red 120–agarose column was concentrated 35-fold to 20 mL, and, in the case of latent preparations, the collagenase was activated by incubation with 0.5 mM PCMB at 4 °C for 30 min. The sample was then applied at 15 mL/h to the affinity column (1.5 × 12 cm) which had previously been equilibrated with 10 mM Tris, 10 mM $CaCl_2$, and 1 M NaCl, pH 7.5, containing 0.05% Brij-35 and 0.1 mM PCMB. The column was washed with the same buffer until the absorbance reached base line, as shown in Figure 2 for an active sample, to give fraction A. In the case of a latent sample, the column was next washed with the same buffer without any PCMB until all of the PCMB was removed, as indicated by the absorbance. Bound protein was then eluted with 100 mM Tris, 100 mM $CaCl_2$, and 1 M NaCl, pH 9.0, containing 0.05% Brij-35 to give fraction B. Zinc ions were omitted in the buffers so that they would not compete with HNC for the hydroxamate ligand. Immediately after collection, the pH of fraction B was adjusted to 7.5 with 0.5 Mes, pH 6.0, containing 1 M NaCl and 0.05% Brij-35, and the $ZnSO_4$ concentration was restored to 50 μM.

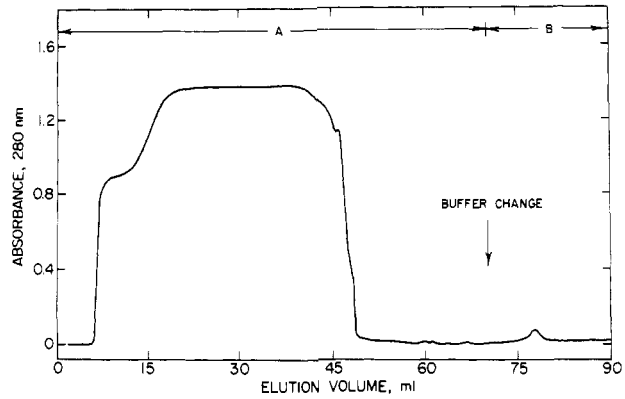

FIGURE 2: Chromatography of Reactive Red 120–agarose column fraction B containing latent HNC over Sepharose-CH–Pro-Leu-Gly-NHOH. The sample was applied to the column and eluted as described in the text.

This step resulted in 62- and 160-fold purifications of the latent and active enzymes with yields of 65 and 120%, respectively. The only detectable impurity in fraction B was a small amount of HNG (see below). It should be noted that the HNC that eluted in fraction B from the latent preparation *remained latent*, even though it was activated by PCMB prior to applying it to the affinity resin. Thus, latent HNC can be *reversibly activated* by PCMB. This affinity resin is also useful for separating latent and active HNC, since in the absence of PCMB, only active HNC binds. This step has been performed over 30 times with a yield in fraction B that ranged from 60 to 120%.

Gelatin–Sepharose Chromatography. Gelatin–Sepharose chromatography was carried out to remove the contaminating HNG by a slight modification of the procedure of Hibbs et al. (1985). Fraction B from the affinity column was diluted 2-fold with a 1 M NaCl solution containing 0.05% Brij-35 to lower the concentrations of Tris and $CaCl_2$. The latent or active sample was then applied to a gelatin–Sepharose column (1 × 10 cm) at a flow rate of 15 mL/h (elution profile not shown). The column was washed with 50 mM Tris, 1 M NaCl, and 5 mM $CaCl_2$, pH 7.5, containing 0.05% Brij-35 and 50 μM $ZnSO_4$ until the absorbance reached the base line to give fraction A, which contains pure HNC. The HNG was eluted with 50 mM Tris, 50 mM $CaCl_2$, and 1 M NaCl, pH 7.5, containing 0.05% Brij-35 and 50 μM $ZnSO_4$, 5% dimethyl sulfoxide, and 10% glycerol. The HNG in fraction B was recovered after dialysis versus 10 mM Tris, 5 mM $CaCl_2$, and 1 M NaCl, pH 7.5, containing 0.05% Brij-35 and 50 μM $ZnSO_4$. As seen from Tables I and II, this step effectively separates both active and latent HNC from the residual, contaminating HNG. Overall, the three-step purification described above gives chromatographically pure 58-kDa latent or active HNC with a typical cumulative yield of 40–80%.

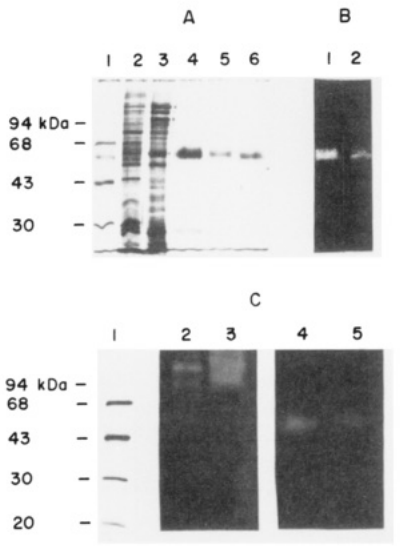

FIGURE 3: (A) SDS–PAGE gel run in the presence of 2-mercaptoethanol showing various chromatographic fractions in the purification of HNC. Lane 1 contains molecular weight standards (40 ng of each). Lanes 2–5 contain the crude buffy coat extract (5 μg), Reactive Red 120–agarose fraction B (2 μg), Sepharose-CH–Pro-Leu-Gly-NHOH fraction B (400 ng), and gelatin–Sepharose fraction A (100 ng) from the purification of latent HNC. Lane 6 is gelatin–Sepharose fraction A (200 ng) from the purification of active HNC. (B) Collagen zymogram showing the collagenase activity associated with purified (gelatin–Sepharose fraction A) latent (lane 1, 200 ng) and active (lane 2, 200 ng) HNC. The zymograms were developed for 24 h at 37 °C. (C) Gelatin zymogram showing the gelatinase activity associated with Sepharose-CH–Pro-Leu-Gly-NHOH fraction B from the latent (lane 2, 200 ng) and active (lane 3, 200 ng) purification of HNC, and purified (gelatin–Sepharose fraction A) latent (lane 3, 4 μg) and active (lane 4, 4 μg) HNC. Molecular weight standards (40 ng of each) are shown in lane 1 for reference. The zymograms were developed for 28 h at 37 °C (lanes 2 and 3) and 41 °C (lanes 4 and 5), respectively.

Yield, Latency, and Storage of HNC. This purification scheme yields 2 mg of latent HNC from 30 units of outdated blood (approximately 5×10^{10} neutrophils). The yield of active HNC is considerably lower, probably due to its destruction by endogenous elastase and cathepsin G (Murphy et al., 1982). Immediately after isolation, "latent" HNC samples are greater than 90% latent. Immediate freezing and storage at −70 °C are required to prevent spontaneous activation of latent HNC, or autolytic degradation and loss of activity of both active and latent HNC. Both forms can be stored frozen at −70 °C for 2 years without appreciable loss in activity. Storage at −10 to −20 °C results in a slow loss in activity with concomitant formation of a 27-kDa degradation fragment. Frequent freeze–thawing of enzyme samples also results in loss of activity, and storage of the enzymes in appropriate aliquots is recommended.

Analysis of Chromatographic Fractions and Purified Enzymes by Gel Zymography and SDS–PAGE. Several of the chromatographic fractions from the purification scheme described above, including gelatin–Sepharose fraction A that contains the purified HNC samples, have been analyzed by SDS–PAGE and by collagen and gelatin zymography (Figure 3A–C). Lane 1 from the SDS–PAGE gel shown in Figure 3A contains low molecular weight standards. Lanes 2–5 contain the crude buffy coat extract, Reactive Red 120–agarose fraction B, hydroxamate affinity resin fraction B, and gelatin–Sepharose fraction A, all from the latent preparation, respectively, while lane 6 contains gelatin–Sepharose fraction A from an active preparation. Inspection of lanes 2 and 3 shows that, even though the Reactive Red 120–agarose column removes a substantial amount of contaminating hemoglobin, fraction B still contains numerous impurities. However, most of these are removed by the Sepharose-CH–Pro-Leu-Gly-NHOH affinity column (lane 4), which is the key step in this purification scheme.

Even though it is not visible on the SDS–PAGE gel, the hydroxamate column fraction B for latent HNC (lane 4, Figure 3A) preparations still contains some HNG. The HNG in small (~200 ng) samples of both latent and active hydroxamate affinity resin fraction B samples is clearly visualized by gelatin zymography as a ladder of bands starting with a molecular weight of 92K (lanes 2 and 3, Figure 3C) (Hibbs et al., 1985). This activity is *not* present in much larger samples (4 μg) of gelatin–Sepharose fraction A for latent and active HNC (lanes 4 and 5, Figure 3C), indicating that this HNG was removed by the gelatin–Sepharose step. As indicated in Tables I and II, a small amount of residual gelatinolytic activity remains associated with the HNC in gelatin–Sepharose fraction A for both the latent and active samples. This gelatinolytic activity is not removed by a second round of gelatin–Sepharose chromatography. Gelatin zymograms containing large quantities of these purified HNC samples (lanes 4 and 5, Figure 3C) show that this residual activity is associated with the HNC bands. Chromatographically pure native and recombinant HFC also exhibit this activity in gelatin zymograms (Fields et al., 1990) which is attributable to the *inherent* gelatinolytic activity of these collagenases (Welgus et al., 1982).

Both purified latent and active HNC exhibit a major band at approximately 58 kDa in the SDS–PAGE gels run either in the presence (lanes 5 and 6, Figure 3A) or in the absence (not shown) of 2-mercaptoethanol. Because these bands are rather broad and diffuse, it is difficult to determine whether latent and active HNC have the same mobilities. However, active HNC appears to reproducibly migrate with a slightly lower (<1–2K) molecular weight. This observation, coupled with the fact that activation during extraction is blocked by PMSF, is consistent with its activation have arisen via proteolysis by a serine proteinase. It must be underscored, however, that this does not in any way imply that this is a physiological means of activation, since the extraction procedure releases enzymes that might never have access to one another under physiological conditions. Proof that the 58-kDa bands in the SDS–PAGE gels are responsible for the collagenase activity observed (Tables I and II) comes from the collagen zymograms shown in Figure 3B. Lanes 1 and 2 contain the same purified latent and active HNC samples shown in lanes 5 and 6 of Figure 3A, respectively. These zymograms show that the collagenase activity is associated solely with the 58-kDa HNC bands. A trace amount of the 75-kDa form of HNC is also barely discernible on these SDS–PAGE gels (Figure 3A). For both latent and active HNC, a ~27-kDa band forms as a function of time (e.g., lane 4, Figure 3A), even when the enzyme is stored frozen at −10 °C. This is an autolytic degradation product, similar to that observed for HFC (Birkedal-Hansen et al., 1988).

These and other studies in our laboratory confirm the observation by Hasty and associates (Hasty et al., 1986) that the two major forms of HNC have molecular weights of 58K and 75K. It is of interest to reconcile these observations with those of others who have claimed molecular weights for latent HNC in the vicinity of 90–105K that decrease by approximately 25K on activation (Murphy et al., 1980; Macartney & Tschesche, 1983). While there is now agreement that HNC is a single polypeptide chain (Knäuper et al., 1990), Macartney

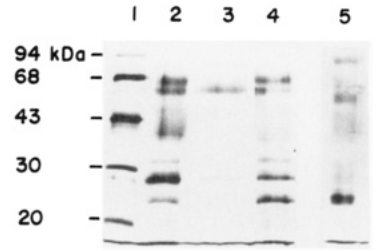

FIGURE 4: SDS–PAGE gel containing chromatographic fractions from an active HNC purification run in the presence (lanes 1–4) and absence (lane 5) of 2-mercaptoethanol. Lanes 1–4 contain molecular weight standards (40 ng of each), Sepharose-CH–Pro-Leu-Gly-NHOH fraction B (1 μg), and gelatin–Sepharose fractions A (100 ng) and B (800 ng), respectively. Lane 5 also contains gelatin–Sepharose fraction B (800 ng).

and Tschesche (1983) originally reported that latent HNC is a 91-kDa disulfide-bonded complex between a 64-kDa active collagenase and a 22-kDa inhibitor. The inhibitor was thought to be released by disulfide exchange to liberate the active collagenase. In the course of purification of *active* HNC described above, a series of observations were made that resembled those first reported by Macartney and Tschesche (1983). When fraction B from the hydroxamate affinity resin from an *active* preparation was subjected to SDS–PAGE under reducing conditions, impurities with approximate molecular weights of 65K and 22K were clearly visible in addition to the 58-kDa HNC band, some 27-kDa HNC fragment, and other minor impurities (lane 2, Figure 4). Chromatography over gelatin–Sepharose separates most of the 58-kDa HNC (fraction A; lane 3 of Figure 4) from the 65- and 22-kDa bands and other impurities (fraction B; lane 4 of Figure 4). Interestingly, under nonreducing conditions, the 65-kDa band present in gelatin–Sepharose fraction A is absent, and a new band with a molecular weight of ~94K is apparent (lane 5, Figure 4). This 94-kDa band is apparently a disulfide-bonded complex of 65- and 22-kDa chains and may be the species observed earlier (Macartney & Tschesche, 1983). The disappearance of the 22-kDa band in lane 5 of this gel cannot be confirmed, since the 27-kDa fragment migrates with an apparent molecular weight of 23K under reducing conditions and obscures it.

Since the 94-kDa fragment is removed by the gelatin–Sepharose column along with the gelatinase activity (Table I), this species is almost surely the 92-kDa HNG described by Hibbs et al. (1985) that has undergone a proteolytic scission. Thus, a serine proteinase released during the extraction is apparently responsible for the hydrolysis of 92-kDa HNG into the 65- and 22-kDa fragments. This clipped 92-kDa HNG species may have been mistaken for HNC, since a sample containing predominantly this HNG by mass, but with a small amount of latent HNC, would appear to activate its HNC collagenase activity in a manner that correlated with the change in molecular weight of the HNG bands. Since PMSF was not included in the extraction buffer in earlier studies (Macartney & Tschesche, 1983), the HNG present may well have undergone the same proteolysis observed here.

Specific Collagenase Activities of Purified HNC. The specific activities of purified active and PCMB-activated latent HNC were determined by using both soluble and fibrillar rat type I collagen as substrates for comparison with the values reported in the literature. The same activities were also measured for trypsin-activated HFC (Table III). The specific activities of active and PCMB-activated latent HNC toward soluble collagen are 7200 and 12000 μg min^{-1} mg^{-1} and toward collagen fibrils are 4200 and 7000 μg min^{-1} mg^{-1}, respectively.

Table III: Specific Activity of Latent and Active HNC toward Rat Type I Collagen

	sp act. (μg min^{-1} mg^{-1})	
enzyme	soluble assay	fibril assay
latent HNC[a]	12000	7000
active HNC	7200	4200
HFC[b]	750	1100

[a] Activated with 0.1 mM PCMB. [b] Activated with trypsin.

These values are at least 10-fold higher than the highest values reported previously for this enzyme (Hasty et al., 1987b) which had been purified by immunoaffinity chromatography by a procedure that involves desorption of the enzyme with 3 M NaSCN. This protocol, which the investigators acknowledged was harsh, activates latent HNC and also apparently lowers the specific activity of the resultant active enzyme. Thus, while these workers found HNC to have a lower specific activity than HFC, we find that the opposite is true. HNC is also more active toward rat tendon type I collagen than trypsin-activated HFC (750 and 1100 μg min^{-1} mg^{-1} for soluble collagen and fibrils, respectively). These activities are considered in more detail in the following paper (Mallya et al., 1990).

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Registry No. Collagenase, 9001-12-1.

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