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Purification of Human Collagenases with a Hydroxamic Acid Affinity Column

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ABSTRACT: Human collagenase has been isolated from skin fibroblasts and rheumatoid synovium by using an affinity matrix, prepared by coupling Pro-Leu-Gly-NHOH to agarose. Following the methodology described herein, the skin enzyme was isolated in two steps in 76% yield and the synovial enzyme was purified in three steps in 71% yield. Importantly, each enzyme hydrolyzed collagen into $^3/_4$–$^1/_4$ cleavage fragments, indicating that a true collagenase had been isolated. The column was specific for the human enzyme since the collagenase from *Clostridium histolyticum* did not bind. The affinity ligand was designed according to the formalism proposed by Holmquist and Vallee [Holmquist, B., & Vallee, B. L. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6216] that effective metalloenzyme inhibitors can be synthesized by coupling a suitable metal-coordinating group to a substrate analogue. In this case, the hydroxamic acid probably coordinates to the active-site metal and the Pro-Leu-Gly moiety is similar to the carboxyl side of the cleavage site of collagen, the enzyme's substrate. The IC_{50} for *N*-(benzyloxycarbonyl)-Pro-Leu-Gly-NHOH is 4×10^{-5} M for both enzymes. The affinity chromatographic procedures described here should aid in future studies on vertebrate collagenases.

Collagenase is a highly specific, neutral protease that cleaves undenatured collagen at a point three-fourths the distance from the amino-terminal end. There is increasing evidence that the enzyme plays a critical role in a variety of normal and pathogenic states, such as resorption of the postpartum uterus (Jeffrey & Gross, 1970), wound healing (Grillo & Gross, 1967), rheumatoid arthritis (Evanson et al., 1967; Werb et al., 1977), and tumor invasion (Liotta et al., 1983). Even though the importance of collagenase is now well recognized, there have been few attempts to regulate its activity because of the absence of structure–function studies that could be used for the rational design of inhibitors. These studies have not been performed for two reasons. First, the enzyme is syn-

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thesized in only small quantities by cells in culture so that there is no rich source of starting material. Second, collagenase is difficult to purify because of contaminating serum proteins and the presence of protein inhibitors that mask its activity in conventional enzyme assays (Welgus et al., 1979).

The supply problem has been solved by the observation that SK hepatoma tumor cells produce a soluble factor that induces cells either to increase collagenase production or to initiate collagenase synthesis (unpublished data). For example, SK hepatoma conditioned media containing this "collagenase-inducing factor" caused skin fibroblasts to produce at least 1000 times more enzyme than those cultured in normal media. Treatment of collagenase-producing cells with this tumor-conditioned media, then, results in medium highly enriched in collagenase.

In this work, a series of peptide hydroxamic acids was synthesized, and one of these, Z-Pro-Leu-Gly-NHOH,[1] was shown to be an effective inhibitor of the enzyme from either human synovial cells or human skin cells. Moreover, when the blocking group was removed and the peptide was covalently bound to agarose, a highly effective affinity resin resulted. By use of the enriched starting material described above and this affinity resin, the present study describes in detail a rapid, two-step isolation scheme for the purification of large quantities of human collagenases.

Materials and Methods

Chemicals. Glycinehydroxamic acid, benzyl succinate, and amastatin were purchased from Sigma. Zincov was purchased from Calbiochem, and captopril was synthesized as described elsewhere (Cushman et al., 1977).

Hydroxamic acids were synthesized by the nucleophilic attack of hydroxylamine on either peptide methyl esters or succinimide esters. Compounds were characterized by amino acid analysis after acid hydrolysis, TLC (silica gel 60 F-254; $CHCl_3$:CH_3OH, 3:1), melting point, IR spectroscopy (1% KBr pellets), and extinction coefficient of the corresponding Fe^{3+} complex at 540 nm (50 μL of 10 mg/mL solution in DMF added to 3.0 mL of 2% $FeCl_3$ in 0.10 N HCl).

Method A. In a clean dry beaker, 1.6 g (5 mmol) of Z-Leu-Gly (Sigma) and 0.75 g (5 mmol) of *N*-hydroxysuccinimide were dissolved in 25 mL of dioxane, and 1.03 g (5 mmol) of DCC was added with stirring. The solution was stirred overnight, and the following day the solid DCU was filtered off. A solution of hydroxylamine was prepared by dissolving 0.52 g (7.5 mmol) of NH_2OH·HCl in 10 mL of dimethylformamide and adding 1.03 mL (7.5 mmol) of triethylamine. After the hydrochloride salt was filtered off, the two solutions were mixed and stirred overnight at room temperature. The next day the solution was neutralized, the solvent removed on the rotovap, and the solid recrystallized from ethyl acetate–hexane. The oil was collected and triturated with ether: yield 500 mg; mp 110–113 °C; amino acid analysis Gly 1.00, Leu 1.03; R_f 0.80; ϵ_{540} = 890.

Method B. In a small beaker, 0.34 g (4.8 mmol) of NH_2OH·HCl was dissolved in 0.75 mL of H_2O and 0.60 mL of ethanol. When all the solid dissolved, the solution was placed in an ice bath and 1 mL of 10 N KOH (10 mmol) was added dropwise with stirring. The ice was removed, and the mixture was added to a solution of 1.4 g (4.8 mmol) of Z-Ala-Gly-OMe (Sigma) in 30 mL of methanol. The solution was stirred for 1 h at room temperature and the pH lowered to below 7 by the dropwise addition of concentrated HCl. The beaker was cooled on ice, the salt was filtered off, and then all the solvent was removed on the rotovap. This solid was taken up in water and extracted with ethyl acetate. The water layer was evaporated on the rotovap and the solid recrystallized from ethyl acetate: yield 400 mg; mp 144.5–146.5 °C; amino acid analysis Gly 1.00, Ala 0.98; R_f 0.70; ϵ_{540} = 890.

N-(Benzyloxycarbonyl)glycinehydroxamic Acid (Z-Gly-NHOH). This compound was prepared from Z-Gly (4.2 g, 20 mmol) by using method A. After the hydoxamic acid was neutralized with acid, the solvent was removed and the solid was recrystallized from hot water: yield 2.2 g; mp 120.5–122 °C; R_f 0.74; ϵ_{540} = 890.

N-(Benzyloxycarbonyl)glycylglycinehydroxamic Acid (Z-Gly-Gly-NHOH). This compound was prepared from Z-Gly-Gly (2.66 g, 10 mmol) by using method A and recrystallized from boiling water: yield 1.6 g; mp 150–151.5 °C; R_f 0.58; ϵ_{540} = 900.

N-(Benzyloxycarbonyl)-L-phenylalanylglycinehydroxamic Acid (Z-Phe-Gly-NHOH). Z-Phe (3.0 g, 10 mmol) and *N*-hydroxysuccinimide (1.15 g, 10 mmol) were dissolved in 40 mL of cold dimethylformamide, and DCC (2.06 g, 10 mmol) was added with rapid stirring. The solution was stirred for 1 h on ice and then overnight at room temperature. The next day the solid DCU was filtered off and the clear solution was added to an aqueous solution of glycine methyl ester, prepared by dissolving the hydrochloride salt (1.78 g, 11 mmol) in water containing $NaHCO_3$ (1.85 g, 22 mmol). The mixture was stirred for 2 h at room temperature, the pH adjusted to 2, the solvent removed, and the solid taken up in ethyl acetate. The solution was extracted with water, the organic layer dried over $MgSO_4$, and the peptide crystallized by adding hexane to the boiling ethyl acetate solution: yield 3.0 g; mp 116.5–118 °C; R_f 0.94; amino acid analysis Gly 1.00, Phe 1.01. The hydroxamate was prepared from this ester (2.1 g, 5 mmol) by using method B. The crude solid was taken up in ethyl acetate and recrystallized from ethyl acetate–hexane: yield 1.0 g; mp 148–150 °C; R_f 0.82; amino acid analysis Gly 1.00, Phe, 1.02; ϵ_{540} = 1013.

N-(Benzyloxycarbonyl)-L-methionylglycinehydroxamic Acid (Z-Met-Gly-NHOH). This compound was prepared from Z-Met-Gly-OEt (Sigma; 2.76 g, 7.5 mmol) by using method B. The crude solid was extracted with ethyl acetate and recrystallized from ethyl acetate: yield 1.0 g; mp 133–134 °C; R_f 0.76; amino acid analysis Gly 1.00, Met 0.73 (starting material Gly 1.00, Met 0.60); ϵ_{540} = 870.

N-(Benzyloxycarbonyl)-L-leucyl-L-leucylglycinehydroxamic Acid (Z-Leu-Leu-Gly-NHOH). Z-Leu (2.7 g, 10 mmol) and *N*-hydroxysuccinimide (1.15 g, 10 mM) were dissolved in dioxane, and DCC (2.06 g, 10 mmol) was added with rapid stirring on ice. The solution was stirred overnight at room temperature, and the next day the solid DCU was filtered off. The clear solution was added to an aqueous solution of Leu-Gly (Sigma; 2.07 g, 11 mmol) prepared by adding the solid to $NaHCO_3$ (1.85 g, 22 mmol) dissolved in water. The mixture was stirred for 4 h at room temperature, the solution adjusted to pH 2 by adding concentrated HCl, and all the solvent removed on the rotovap. The gummy mass was dissolved in ethyl acetate and extracted with water, and the organic layer was dried over $MgSO_4$. The peptide was recrystallized from ethyl acetate–hexane. The oil that formed was then collected and crystallized by triturating with ether: yield 2.1 g; mp 96.5–99.5 °C. The hydroxamate was prepared from Z-Leu-

[1] Abbreviations: Z, benzyloxycarbonyl; DCC, dicyclohexylcarbodiimide; DCU, dicyclohexylurea; EDTA, ethylenediaminetetraacetate; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; TLC, thin-layer chromaography; DEAE, diethylaminoethyl; DMF, dimethylformamide.

Leu-Gly (1.8 g, 4.1 mmol) by using method A: yield 0.9 g; mp 113–116 °C; amino acid analysis Gly 1.00, Leu 2.04; R_f 0.81; ϵ_{540} = 930.

N-(Benzyloxycarbonyl)-L-prolyl-L-leucylglycinehydroxamic Acid (Z-Pro-Leu-Gly-NHOH). Z-Pro-OSu (3.46 g, 10 mmol) was prepared as described previously (Anderson et al., 1964), dissolved in dioxane, and added to an aqueous solution of Leu-Gly (1.88 g, 10 mmol) and sodium bicarbonate (1.68 g, 20 mmol). The solution was stirred overnight at room temperature and acidified to pH 2.0, and all the solvent was removed under reduced pressure. The solid was washed with water and recrystallized from ethanol–water: yield 3.2 g; mp 165.5–166 °C. In a clean, dry beaker, Z-Pro-Leu-Gly (2.72 g, 6.5 mmol) and *N*-hydroxysuccinimide (0.98 g, 6.5 mmol) were dissolved in 25 mL of dioxane, and DCC (1.34 g, 6.5 mmol) was added with stirring. The solution was stirred overnight, and the following day the solid DCU was filtered off. A solution of NH_2OH·HCl (0.68 g, 9.75 mmol) was prepared by dissolving the solid in 10 mL of dimethylformamide and adding triethylamine (1.45 mL, 9.75 mmol). After the hydrochloride salt was filtered off, the two solutions were mixed and stirred overnight at room temperature. The next day the solution was neutralized, the solvent removed on the rotovap, and the solid recrystallized from ethanol–water: yield 1.3 g; mp 141–143 °C; R_f 0.63; amino acid analysis Gly 1.00, Leu, 1.04, Pro 0.95; ϵ_{540} = 910.

Collagenase Affinity Column. Five hundred milligrams of Z-Pro-Leu-Gly hydroxamate were treated with anhydrous HF to remove the carbobenzyloxy blocking group. The unblocked peptide was dissolved in water and extracted 2 times with chloroform and once with hexane, and the aqueous layer was lyophilized. Prolylleucylglycinehydroxamic acid was coupled to activated CH-Sepharose 4B according to the recommended Pharmacia procedure. Specifically, the freeze-dried resin (15 g) was swollen and washed with 3 L of 1 mM HCl on a sintered glass funnel to give 45 mL of gel with a capacity of 5–7 μmol/mL. The unblocked peptide (180 mg) was dissolved in 45 mL of 0.01 M sodium bicarbonate, pH 8.0, and mixed with the gel for 60 min at 23 °C. The coupled gel was then washed with 0.10 M Tris and 0.50 M NaCl, pH 8.0, alternating with 0.10 M sodium acetate and 0.50 M NaCl, pH 4.0, and stored at 4 °C in 0.05 M Tris, 0.50 M NaCl, and 0.01 M $CaCl_2$, pH 7.5. A 0.50-mL aliquot of the gel was hydrolyzed in 6 N HCl and found to contain 2.5 μmol each of Pro, Leu, and Gly.

[^{14}C]Collagen. [^{14}C]Collagen was prepared by reductive methylation of calf skin collagen at 4 °C using [^{14}C]formaldehyde and sodium borohydride. Calf skin collagen (Sigma) was dissolved at 7.5 mg/mL in 60 mL of 0.10 M acetic acid and dialyzed at 4 °C against 0.15 M potassium phosphate, pH 7.6, for 8 h, followed by dialysis overnight against 0.40 M NaCl. The collagen solution was then adjusted to pH 9.0 by addition of 0.50 M sodium borate, and then 1 mCi of [^{14}C]formaldehyde (10 mCi/mmol) was added. After 1 min, 0.10 M sodium borohydride (660 μL in 1.3 mM NaOH) was added in four aliquots, followed by an additional aliquot (340 μL) 30 min later. The solution was then dialyzed exhaustively against 0.01 M acetic acid, centrifuged to remove particulates, and stored frozen in 1-mL aliquots. The specific activity was 1.3×10^6 dpm/mg. Unlabeled collagen was prepared in the same way, stored frozen, and mixed with labeled collagen at a ratio of 9 to 1. For assay, the diluted [^{14}C]collagen was dialyzed 6–8 h at 4 °C against 0.15 M potassium phosphate, pH 7.6, followed by dialysis overnight against 0.40 M sodium chloride. This solution was centrifuged to remove any undissolved collagen and stored at 4 °C.

Enzyme Assays. Collagenase assays (Terato et al., 1976) were performed in 1.5-mL polypropylene microfuge tubes. Each assay tube contained 50 μL of [^{14}C]collagen solution (4 mg/mL) and 50 μL of 1.0 M glucose, 0.10 M Tris, 0.40 M NaCl, and 0.02 M $CaCl_2$, pH 7.5. This solution was incubated for 10 min at 35 °C and the reaction initiated by the addition of 100 μL of enzyme solution. Those samples containing procollagenase were first activated by incubating 100-μL aliquots with 1–5 μL of 10 mg/mL trypsin (in 1 mM HCl) for 20 min at 23 °C, followed by 20 μL of 5 mg/mL soybean trypsin inhibitor (in 0.05 M Tris and 0.01 M $CaCl_2$, pH 7.5) to quench the trypsin activity. The collagenase assay was terminated after 30 min at 35 °C by the addition of 20 μL of 0.08 M 1,10-phenanthroline in 50% (v/v) dioxane, and the incubation was continued for 1 h at 35 °C to denature the collagen digestion products. Each sample was cooled for 15 min at 23 °C and 200 μL of dioxane added with vigorous vortexing to precipitate uncleaved collagen. Following centrifugation at 11 000 rpm, 350-μL aliquots were added to 5.0 mL of Pico-Fluor 30 to determine radioactivity.

Thermolysin activity was measured with the chromophoric substrate *N*-(furylacryloyl)glycyl-L-leucinamide (Feder, 1968). In a typical experiment, 10 μL of enzyme solution was added to 3.0 mL of 1 mM substrate dissolved in 0.10 M NaCl, 0.01 M $CaCl_2$, and 0.05 M Tris, pH 7.5, and the decrease in absorbance monitored at 345 nm with a Gilford spectrophotometer.

Collagenase Inhibition. Collagenase activities were measured in the presence of inhibitors by adding an aliquot of inhibitor (0–85 μL) and buffer to 100 μL of glucose–collagen solution to give a total volume of 185 μL, incubating at 35 °C for 10 min, and then initiating the reaction with 15 μL of purified collagenase. The rest of the assay was performed as described above. To determine the IC_{50} value, inhibition was measured over a 10-fold concentration range at 10 μM collagen.

To determine the mechanism of inhibition, the equation used was (Holmquist & Vallee, 1974)

$$\log (A_0/A_1 - 1) = \log K_1 + n \log I \qquad (1)$$

where A_0 is the activity in the absence of inhibitor and A_1 is the activity in the presence of inhibitor.

Cell Culture. All cells were cultured by using standard cell culture techniques.

Amino Acid Analysis. Protein samples were dialyzed against 0.1 N HCl, lyophilized, and hydrolyzed with 6 N HCl for 20 h in sealed tubes at 110 °C. Analyses were performed on a Beckman 119CL amino acid analyzer.

Gel Electrophoresis. Polyacrylamide electrophoresis with sodium dodecyl sulfate was performed in 10% acrylamide according to the method of Laemmli (1970).

RESULTS

Inhibition Studies. While there have been no definitive studies on the metal content of mammalian collagenase, virtually all preparations have been sensitive to metal-chelating agents, implying that metals are essential for activity. On the basis of these observations, the activity of the enzyme was measured in the presence of the following inhibitors, which have been effective toward other metalloenzymes: L-benzyl succinate (carboxypeptidase), amastatin (leucine aminopeptidase), captopril (angiotensin converting enzyme), Zincov (thermolysin), and phosphoramidone (thermolysin). Only Zincov, which contains a hydroxamic acid moiety, showed meaningful inhibition (data not shown). Therefore, a series

Table I: IC_{50} Values of Hydroxamic Acid Inhibitors of Collagenase[a]

inhibitor	IC_{50} (mM) synovial	fibroblast
Z-Gly-NHOH	0.96	0.60
Gly-NHOH	18.0	>1
Z-Gly-Gly-NHOH	2.0	3.0
Z-Ala-Gly-NHOH	2.6	1.3
Z-Leu-Gly-NHOH	0.17	0.48
Z-Phe-Gly-NHOH	0.10	0.15
Z-Met-Gly-NHOH	0.20	0.15
Z-Leu-Leu-Gly-NHOH	0.33	0.30
Z-Pro-Leu-Gly-NHOH	0.047	0.040

[a] 0.50 M NaCl, 0.01 M $CaCl_2$, 0.05 M Tris, pH 7.5, 35 °C.

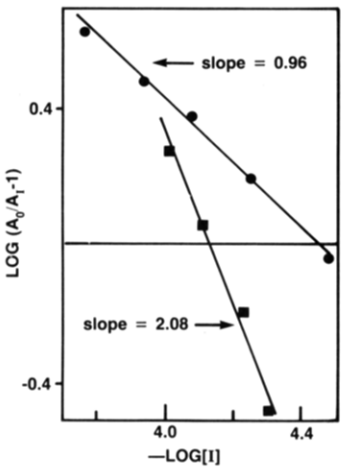

FIGURE 1: A plot of eq 1 (see text) of inhibition data for 1,10-phenanthroline (■) and Z-Pro-Leu-Gly-NHOH (●). The intersection with the x axis gives the K_I value, and the slope is the average number of moles of inhibitor bound per mole of enzyme.

of hydroxamic acids was synthesized and tested for collagenase inhibition.

Since the hydroxamate functional group is an essential component for inhibition, a series of peptide hydroxamic acids was synthesized to probe those features of the peptide chain that lead to most effective inhibition (Table I). Following the notation of Schechter and Berger (1967), each peptide had glycine in position P_1. In addition, each peptide contained a blocked N-terminus since the positive charge of the free amino group lowered the observed inhibition constant. Thus, the IC_{50} value for Gly-NHOH was about 25 times greater than that for Z-Gly-NHOH. In the peptide series Z-X-Gly-NHOH, those peptides containing bulky, hydrophobic side chains (X = Leu, Phe, Met) in position P_2 had IC_{50} values 10–20 times lower than those with small aliphatic side chains (X = Ala, Gly). The P_3 position was examined by comparing the IC_{50} value for proline in this position to that for the corresponding leucine-containing peptide, Z-X-Leu-Gly-NHOH (X = Leu, Pro). With an IC_{50} of 4×10^{-5} M, this proline-containing peptide was 10 times more effective than the corresponding leucine analogue, and moreover, this was the most effective inhibitor found.

The mechanism of Z-Pro-Leu-Gly-NHOH inhibition was determined by plotting inhibition data according to eq 1. As shown in Figure 1, the slope of the line is 1, indicating the formation of a ternary complex. For comparison, inhibition by 1,10-phenanthroline was plotted in the same way. In this case, the slope of the line is greater than 2, indicating that inhibition occurs by metal removal.

Affinity Column. As described under Materials and Methods, Pro-Leu-Gly-NHOH was covalently bound to agarose as an affinity ligand. In a typical experiment, 100

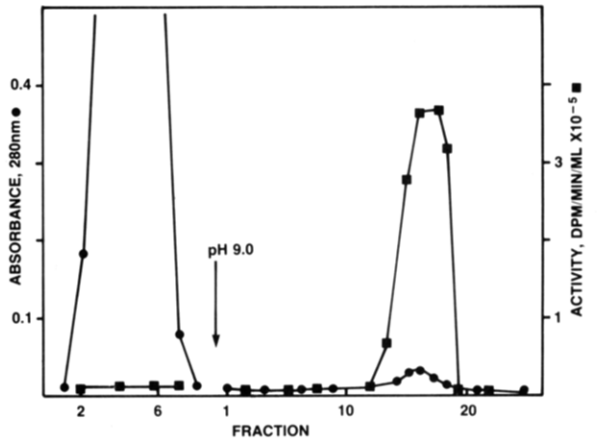

FIGURE 2: Affinity chromatography of human skin collagenase. Details of column conditions are given under Results.

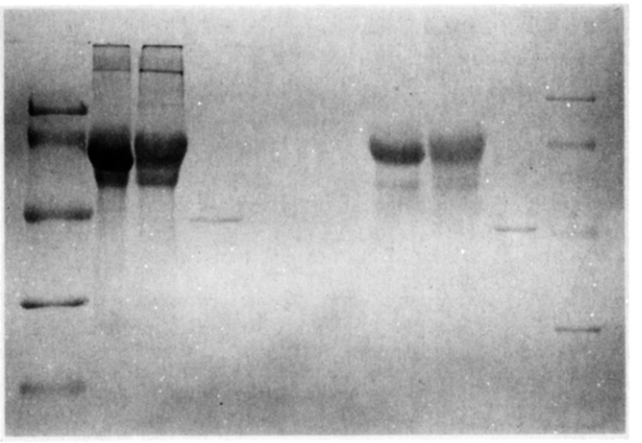

FIGURE 3: SDS (10%) gels of collagenase purification. Lane 1, standards 94 000, 67 000, 43 000, 30 000, and 21 000; lane 2, starting synovial media; lane 3, ammonium sulfate fraction; lane 4, purified human synovial collagenase; lane 5, starting skin media; lane 6, ammonium sulfate fraction; lane 7, purified skin collagenase; lane 8, standards.

mL of SK hepatoma medium was removed from either skin fibroblasts or synovial fibroblasts and treated with ammonium sulfate to 55% saturation. The precipitate was collected by centrifugation, dissolved in water, and then dialyzed against 0.50 M NaCl, 0.01 M $CaCl_2$, and 0.01 M Tris, pH 7.5. Most cells produce collagenase as an inactive zymogen or as an enzyme–inhibitor complex so that little or no activity is observed in the starting cell culture medium. However, when this same medium is treated with trypsin, collagenase activity is generated. Therefore, the dialyzed protein solution was made 1 mg/mL in trypsin and allowed to stand at room temperature for 20 min, and then solid soybean trypsin inhibitor was added to give a final concentration of 1.5 mg/mL. The mixture was then pumped onto the affinity column (1.5 × 15 cm) at 15 mL/h and the column washed with 0.50 M NaCl, 0.01 M $CaCl_2$, and 0.01 M Tris, pH 7.5. As shown in Figure 2, most of the protein washed through the column and all the activity was bound. When the protein absorbance returned to zero, the column was washed with 0.50 M NaCl, 0.10 M $CaCl_2$, and 0.10 M Tris, pH 9.0. Since collagenase has lower stability at high pH, 2.5-mL fractions were collected into 1 mL of 0.50 M NaCl and 0.60 M Tris, pH 6.5. Under these conditions, all the activity was eluted in a single protein peak (Figure 2). For the skin enzyme, when the three or four peak fractions were pooled and examined on SDS gels (Figure 3), one major band and a faint minor band were found at molecular weight 45 000 and 50 000, respectively, indicating that

Table II: Purification of Human Collagenases

	skin					synovial				
step	protein (A_{280})	total act.[a] × 10^{-3}	sp act. (units/A_{280}) × 10^{-3}	recovery (%)	purifcn (x-fold)	protein (A_{280})	total act.[a] × 10^{-3}	sp act. (units/A_{280}) × 10^{-3}	recovery (%)	purifcn (x-fold)
media	432	8450	20	100	1	3486	5890	1.7	100	1
ammonium sulfate	92	6290	68	74	3.4	869	2051	2.4	35	1.4
affinity column	0.63	6600	10600	76	530	1	3484	3484	59	2050
AcA 44						0.42	4200	10000	71	5880

[a] Activity expressed as dpm/min.

the enzyme was essentially pure (see below). For the synovial enzyme there were still some impurities remaining after this affinity step so that an additional gel exclusion column was required for complete purification. When the active fractions from this column were pooled and examined on SDS gels, a single band of molecular weight 45 000 was found (Figure 3). The purification scheme for both enzymes is summarized in Table II, and as shown there, both can be isolated in high yield and with high specific activity. Importantly, when either of these enzymes was incubated with collagen and the cleavage products were visualized on SDS gels, only the characteristic $^3/_4$–$^1/_4$ collagen cleavage products were observed (data not shown).

After each run, the column was regenerated by washing with 0.05 M EDTA and 0.50 M NaCl, pH 7.5, followed by 0.50 M NaCl, 0.01 M $CaCl_2$, and 0.01 M Tris, pH 7.5. One column was used over 25 times over a period of 6 months, with no apparent loss of capacity or other chromatographic properties.

Since the affinity resin contains a metal-chelating group, other metalloenzymes can be bound to the column. Therefore, to determine the specificity of the resin, two other Zn^{2+}-containing enzymes—thermolysin and clostridial collagenase—were applied to the column under identical conditions as those employed for the human enzyme. Only thermolysin was bound to the resin, and it could be eluted under the same conditions as those employed above for human collagenase (data not shown).

Discussion

The rapid purification of a wide variety of proteolytic enzymes from diverse sources has been made possible by the development of affinity resins, prepared by covalently linking a small organic inhibitor to an insoluble matrix. The success of this methodology depends critically on the specificity, reversibility, and strength of binding of these small molecules to the protease. Thus Graves and Wu (1974) showed that the inhibitor's dissociation constant must be 10^{-4} M or less so that the protease can bind tightly to the ligand–matrix to allow extraneous proteins to be washed away. On the other hand, the dissociation constant must be sensitive to some mild change in solvent conditions so that it increases to 10^{-2} M or greater to permit the enzyme to be washed off under nondenaturing conditions. Since a search of the collagenase literature indicated that no small, organic inhibitor was known, an affinity ligand had to be designed that possessed the properties described above.

The first step in the design of any inhibitor is to determine if the enzyme of interest is a serine protease, sulfhydryl protease, or metalloprotease. The fact that synovial and fibroblast collagenases were inhibited by 1,10-phenanthroline with a pK_I of 4.1 (Figure 1) indicates that they are most likely metalloenzymes since this kind and magnitude of inhibition are diagnostic of this particular class (Felber et al., 1962; Holmquist & Vallee, 1974; Breddam et al., 1979). Effective inhibitors can be designed for this kind of protease by incorporating a metal-coordinating group onto a peptide moiety that is preferably a substrate analogue, and indeed, there are now numerous inhibitors that exemplify this approach (Holmquist & Vallee, 1979). The various coordinating groups that have been successfully used with other metalloenzymes are summarized in Table III, and to determine which one would work best for collagenase, each of the compounds listed in Table III was tested as a potential inhibitor. Only Zincov showed meaningful inhibition, indicating that hydroxamic acids provided the best opportunity for successful inhibitor design.

Table III: Coordinating Groups That Inhibit Metalloenzymes

inhibitor	coordinating group	representative enzyme
captopril	sulfhydryl	angiotensin converting enzyme (Ondetti et al., 1977)
phosphoramidone	phosphoramidate	thermolysin (Suda et al., 1977)
L-benzyl succinate	carboxylate	carboxypeptidase A (Byers & Wolfenden, 1973)
Zincov	hydroxamate	thermolysin (Hudgin et al., 1981)
amastatin	α-hydroxycarbonyl	leucine aminopeptidase (Nishizawa & Saino, 1977)

Following the formalism developed by Holmquist & Vallee (1979), the structure of the enzyme's native substrate, collagen, was next considered to determine which peptide sequence to synthesize. It has been reported (Dixit et al., 1979) that rheumatoid synovial collagenase cleaves native collagen at a specific site one-fourth the length of the collagen molecule from the carboxyl end in each of the three chains, and the amino acid sequence around this cleavage site in chick and calf skin is

chick (α_2 chain) Hyp-Gly-Pro-Gln-Gly↓Ile-Leu-Gly-

chick (α_1 chain) Pro-Gly-Pro-Gln-Gly-Ile-Ala-Gly-

calf (α_1 chain) Pro-Gly-Pro-Gln-Gly-Ile-Ala-Gly-

From the common occurrence of -Pro-Gln-Gly- on the carboxyl side of the cleavage site, one would predict that Z-Pro-Gln-Gly-NHOH would be an effective inhibitor since it incorporates both the necessary metal-coordinating group and also a portion of the collagen sequence where hydrolysis occurs. However, introduction of glutamine into a peptide chain can be difficult because the γ-amide group is subject to dehydration when the α-carbonyl group is activated with dicyclohexylcarbodiimide (Gish et al., 1956); therefore, the corresponding leucine analogue Z-Pro-Leu-Gly-NHOH was prepared. As shown in Table I, this is an effective inhibitor, giving an IC_{50} value of 4×10^{-5} M.

To confirm further the importance of the Pro-Leu-Gly sequence for effective inhibition, other peptide hydroxamic acids

of various lengths and amino acid substitutions were synthesized and tested. As shown in Table I, those sequences that contain bulky, hydrophobic residues result in better inhibitors, consistent with the hydrophobic nature of the collagen substrate. However, a comparison of the tripeptides Z-Pro-Leu-Gly-NHOH and Z-Leu-Leu-Gly-NHOH shows that hydrophobicity alone does not determine the magnitude of inhibition. Even though both peptides are hydrophobic, the proline-containing peptide is about 10 times more effective than the corresponding leucine analogue. Taken together, then, these data provide additional evidence that the effective inhibition shown by Z-Pro-Leu-Gly-NHOH is not just due to nonspecific hydrophobic interaction but depends on the close similarity between the peptide's sequence and that of the collagen substrate.

For the affinity purification of metalloenzymes, the K_I value and the mode of inhibition determine the usefulness of the inhibitor as an affinity ligand. Two mechanisms are generally proposed for inhibition (Vallee & Wacker, 1970): one in which an inactive ternary complex is formed between enzyme, metal, and inhibitor (A) and a second in which the inhibitor removes the metal from the enzyme, producing inactive apoenzyme (B). For affinity chromatography, compounds fol-

$$\text{E·Zn} + \text{I} \rightarrow \text{E·Zn·I} \quad \text{(A)}$$

$$\text{E·Zn} + n\text{I} \rightarrow \text{E} + \text{ZnI}_n \quad \text{(B)}$$

lowing mechanism A are preferred since the holoenzyme binds to the resin while those following mechanism B are of no use since they are behaving only as metal-sequestering agents. To distinguish between these two mechanisms, the Z-Pro-Leu-Gly-NHOH inhibition data were plotted according to eq 1 and the slope of the line was equal to 1 (Figure 1). Since the value of the slope is equal to the number of inhibitor molecules bound to the enzyme, these data indicate that mechanism A is the probable inhibition mechanism. Thus, this peptide hydroxamic acid is bound to the enzyme and metal as a ternary complex and is not functioning by removing the metal.

The formation of this ternary complex and the tight binding to collagenase indicate that Z-Pro-Leu-Gly-NHOH fulfills the first requirement for an affinity ligand. Nishino and Powers (1979) also found hydroxamic acids to be good inhibitors of thermolysin and attached one of them to an insoluble support as an affinity ligand. Importantly, when the pH was raised to 9.0, the enzyme was washed off the column with no evidence of denaturation. Thus, hydroxamic acids, in general, and Z-Pro-Leu-Gly-NHOH, in particular, satisfy all the requirements for a good affinity ligand. They are tight-binding inhibitors at pH 7.5, and a mild solvent condition exists (pH 9.0) where they lose their binding ability.

Other workers (Eisen et al., 1974) have prepared affinity resins using collagen as the ligand, but these have had only limited success. Thus, collagenase could be obtained by passing crude preparations through collagen–Sepharose, but contaminating proteins were also bound, requiring a number of preliminary purification steps, such as gel filtration and DEAE chromatography. Even when the enzyme preparation was appropriately prepurified, the yield of purified enzyme from the column was low. Attempts to circumvent these problems by using purified α_1 collagen chains or their cyanogen bromide cleaved fragments as ligands have been largely unsuccessful. All these difficulties are undoubtedly due to the small number of collagen molecules that can be bound to the agarose backbone, to the nonspecific hydrophobic binding that occurs between the immobilized collagen and contaminating proteins, and to the harsh conditions that are occasionally used for elution. Importantly, all these problems have been solved here by using Pro-Leu-Gly-NHOH covalently bound through a six-carbon chain to a Sepharose backbone.

Table IV: Amino Acid Composition of Human Collagenases

amino acid	synovial residue/45 000	skin residue/45 000	skin[a] residue/47 500
Asx	53	52	51
Thr	24	23	24
Ser	36	32	25
Glx	46	45	42
Pro	29	26	27
Gly	50	49	36
Ala	31	29	28
$^1/_2$-Cys	0	0	5
Val	11	19	22
Met	6	6	6
Ile	18	19	18
Leu	27	24	26
Tyr	15	17	18
Phe	26	28	29
His	16	11	14
Lys	17	21	20
Arg	20	22	21

[a] From Stricklin et al. (1978).

The utility of this column can best be demonstrated with a few examples. Skin fibroblast collagenase is particularly difficult to isolate because the fibroblast also synthesizes an inhibitor that masks the enzymatic activity in the collagen assay system (Stricklin & Welgus, 1983; Stricklin et al., 1977). In the conventional purification scheme (Stricklin et al., 1977) a CM-Sephadex ion-exchange column was used to separate the inhibitor from the enzyme. In our hands, this step was time-consuming (2–3 days), and there was frequently some inhibitor left in the enzyme preparation because the two emerged at only slightly different salt concentrations on gradient elution from the CM resin. This difficulty is totally avoided with this affinity column. As shown in Figure 2 and summarized in Table II, the skin enzyme can be purified over 500-fold in 76% yield by using this single purification step. When visualized on SDS gels in the presence of mercaptoethanol, active collagenase isolated in this way consists of two bands (Figure 3)—a major species with molecular weight 45 000 and a minor one with molecular weight 50 000. When the affinity-purified material was passed over an AcA 44 gel exclusion column (data not shown), a molecular weight of 45 000 was found, indicating the absence of sulfhydryl-linked subunits.

The properties of the skin enzyme isolated here agree closely with those previously published by Stricklin and co-workers (1978). In that work, the zymogen was first purified and then activated with trypsin. Two zymogen species were found with molecular weights of 60 000 and 55 000 that were converted (by trypsin treatment) into two enzyme products of molecular weight 45 000 and 50 000, values that correspond to those reported here. As shown in Table IV, the amino acid composition of the material isolated here agrees well with that reported by Stricklin (1977). Except for the absence of cysteic acid in the preparation here, the composition is similar, indicating that the same enzyme has been isolated by the two methods.

The affinity column was also used to isolate a collagenase from human synovial cells. Once again, the major purification occurs at the affinity column step (Table II), but this time an additional gel filtration step was occasionally required to achieve complete purification. The molecular weight and amino acid composition of this synovial enzyme agree closely with those for the skin enzyme; however, in this case, only a single species of molecular weight 45 000 was found. The

availability of large quantities of purified synovial collagenase is especially important for structure–function studies because of the prominent role this enzyme plays in tissue destruction associated with rheumatoid arthritis.

The affinity column is specific for human collagenase since the enzyme from *Clostridium histolyticum* did not bind to the column. This result may seem surprising since the bacterial enzyme is also a metalloprotease, containing one Zn^{2+} per mole of protein (Bond & Van Wart, 1984), and it might seem that the hydroxamate would bind to the metal and retain this enzyme. However, even though the human and bacterial enzymes are both metalloenzymes, their substrate specificities, and hence the architecture of their respective active sites, are different. For example, mammalian collagenases cleave the native collagen molecule across the three strands at only one place, giving two pieces that retain their helical structure, while the bacterial enzyme is far less specific, degrading collagen into a large number of small pieces. In addition, the clostridial enzyme cleaves the collagen chain at Y-Gly sequences, producing amino-terminal glycines (Bornstein, 1967), while the mammalian enzyme cleaves at a -Gly-Leu or -Gly-Ile sequence, producing carboxyl-terminal glycines. Thus, while both enzymes act on the same substrate, they achieve collagen degradation by very different means.

While clostridial collagenase does not bind to the column, other bacterial, Zn^{2+}-containing enzymes can bind. Thus, thermolysin was bound and eluted from the column under the same conditions used for human collagenase. Therefore, care must be exercised in the interpretation of results from this affinity column. Cell culture medium contains a number of proteases, such as proteoglycanases (Takagaki & Gross, 1984), some of which may be metalloenzymes that could bind to the column. Elution of these proteins and the observation of [^{14}C]collagen fragments in the collagenase assay would be erroneously interpreted to mean the presence of collagenase in the culture fluid. To verify that a true collagenase has been isolated, SDS gels of enzyme-treated collagen must be run to show the presence of the $^3/_4$–$^1/_4$ cleavage products. In the work presented here, the synovial and skin enzymes showed only these two cleavage products (data not shown).

In conclusion, the affinity chromatographic procedure described here is a general method applicable to collagenases from a variety of human sources. While the focus of this work has been on the large-scale isolation of the enzyme, the column is also useful for the detection of small quantities of enzyme. The column serves two purposes: first, it removes inhibitors that mask enzyme activity, and second, it serves to concentrate the activity, making the detection of collagenase much more sensitive. With the procedure described here, collagenase activity may be found to be more prevalent than previously thought, providing more insight into the role this enzyme plays in tissue remodeling and pathogenic processes.

Registry No. Z-Gly-NHOH, 76960-28-6; Z-Gly-Gly-NHOH, 66179-54-2; Z-Phe-Gly-NHOH, 103478-80-4; Z-Met-Gly-NHOH, 103478-81-5; Z-Leu-Leu-Gly-NHOH, 103478-82-6; Z-Pro-Leu-Gly-NHOH, 103145-74-0; Z-Leu-Gly-NHOH, 103145-73-9; Z-Ala-Gly-NHOH, 93475-77-5; Gly-NHOH, 5349-80-4; Z-Leu-Gly, 2706-38-9; Z-Ala-Gly-OMe, 4840-29-3; Z-Gly-Gly, 2566-19-0; Z-Met-Gly-OEt, 27482-82-2; Z-Gly, 1138-80-3; Z-Phe, 1161-13-3; Gly-OMe, 616-34-2; Z-Phe-Gly-OMe, 4818-07-9; Leu-Gly, 686-50-0; Z-Leu, 2018-66-8; Z-Leu-Leu-Gly, 47688-56-2; Z-Pro-OSu, 103478-83-7; Z-Pro-Leu-Gly, 7801-38-9; collagenase, 9001-12-1; 1,10-phenanthroline, 66-71-7.

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