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PARTIAL PURIFICATION AND CHARACTERIZATION OF A GELATIN-SPECIFIC PROTEASE FROM THE CULTURE MEDIA OF HUMAN PULMONARY ALVEOLAR MACROPHAGES

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A gelatin-specific protease from the culture media of human pulmonary alveolar macrophages has been partially purified by gel filtration and characterized. The macrophages were obtained by bronchopulmonary lavage from the lungs of disease-free smoking volunteers. The gelatin-specific protease initially requires trypsin activation. After chromatographing the culture media on a Sephadex G-200 column, trypsin is no longer required for activation. The gelatin-specific protease reported here shares many properties of previously reported gelatinases. It is inhibited by EDTA, cysteine, dithiothreitol and serum. It is unaffected by other protease inhibitors: phenylmethylsulfonyl fluoride, tosyllysine chloromethyl ketone and *p*-chloromercuribenzoate. Of all substrates tested, activity was observed only with gelatin. It was inactive toward collagen, elastin and methemoglobin. This enzyme may have a role in the digestion of collagen which has been cleaved by a mammalian collagenase.

Introduction

Collagen degradation is an important and sometimes overlooked component of interstitial pulmonary fibrosis [1]. Due to its phagocytic and digestive properties, one of the cells that could be involved in the degradation of lung connective tissue is the pulmonary alveolar macrophage. In a preliminary report, we noted significant collagenolytic activity in the media of cultured human pulmonary macrophages [2] employing a previously described reduced collagen substrate [3]. More detailed evaluation of the substrate for mammalian collagenase, suggests that a portion of the borohydride reduced collagen was denatured during the assay. Thus much, if not all, of the activity we observed was against gelatin and not collagen. The present communication further describes the nature of this activity, which appears to be more correctly designated as a gelatin-specific protease.

Other gelatin-specific proteases from various tissue sources have been reported in the literature. These sources include culture media of rabbit cranial bone explants [4], culture media of rheumatoid synovium [5], human polymorphonuclear leukocytes [6] and rat sperm [7]. The gelatin-specific protease to be reported shares many characteristics of these enzymes; it is inhibited by EDTA and cysteine, and gelatin appears to be its primary substrate.

Materials and Methods

Collection and culture of pulmonary alveolar macrophages. Human pulmonary alveolar macrophages were collected from disease-free volunteers who had smoked at least 20 cigarettes a day for 5 years. They were between 21 and 50 years of age.

Abbreviations: PZ peptide, *p*-phenyl azobenzyloxycarbonyl-(PZ)-L-Pro-L-Leu-Gly-L-Pro-D-Arg; $Suc(Ala)_3$-NA, *N*-succinyl L-alanyl-L-alanyl-L-alanine *p*-nitroanilide.

Institutional review board approved procedures were utilized in obtaining informed consent. Subsegmental bronchopulmonary lavage was done through a fiberoptic bronchoscope using 5 50-ml portions of warm physiological saline according to the method of Reynolds and Newball [8]. The saline-suspended cells were centrifuged (1 000 × *g*) and resuspended in Dulbecco's Modified Eagle Medium with 0.22 g $NaHCO_3$, 100 units penicillin, 100 μg streptomycin and 0.1 mM non-essential amino acids/100 ml. All cell culture supplies were obtained from Grand Island Biological Co. The cells were counted in a hemocytometer and 5×10^6 cells in 5 ml media were plated per 60 × 15 mm round tissue culture dish. A smear was also made of the cells and stained. Examination of the stained cells by light microscopy showed that typically over 99% were macrophages. The cells were allowed to settle for 2 h in an incubator at 37°C in an atmosphere of 5% CO_2. The medium, containing any unattached cells, was removed and replaced with fresh medium as described above containing 0.2% bovine serum albumin (Sigma Chemical).

Collection of media. Every 2–3 days medium was removed and filtered through a 0.22 μm filter (Millipore) to remove dead cells and cell debris. Fresh medium was added to the culture dishes. Cultures were typically maintained for 2 weeks. Collected media were dialyzed against 0.01 M Tris-HCl, pH 7.6, at 4°C overnight, lyophilized to dryness and stored at 4°C until analysis. Before proceeding as described below, the lyophilized media were reconstituted to one-tenth the original volume in 0.05 M Tris-HCl, pH 7.6/0.005 M $CaCl_2$/0.02% NaN_3.

Enzyme assays. Proteases used in the following assays were from these sources: porcine pancreatic elastase prepared by the method of Shotton [9] was a gift from Dr. Philip J. Stone, Department of Biochemistry, Boston University School of Medicine; bovine pancreatic trypsin and porcine stomach mucosa pepsin were purchased from Sigma Chemical; bacterial collagenase was prepared by the method of Hu et al. [3] and partially purified tadpole collagenase was a gift of Dr. Elvin Harper, Dept. of Chemistry, University of California at San Diego.

Enzyme inhibitors and activators were from these sources: cysteine, dithiothreitol, *p*-chloromercuribenzoate, tosyllysine chloromethyl ketone, phenylmethylsulfonyl fluoride and soya bean trypsin inhibitor were purchased from Sigma Chemical. EDTA, NaN_3 and NaCN were purchased from Fisher Chemical. *N*-Acetyl-L-Ala-L-Ala-L-Pro-L-Ala-chloromethyl ketone and *N*-acetyl-L-Ala-L-Ala-L-Pro-L-Val-chloromethyl ketone were gifts from Dr. James Powers, Dept. of Chemistry, Georgia Insitute of Technology.

In procedures requiring the measurement of radioactivity, a Packard Tri-Carb liquid scintillation spectrometer, Model 3255 was used. This instrument measures ^3H at 40% efficiency and ^{14}C at 67% efficiency (Formula 963 cocktail, New England Nuclear).

Gelatinolytic activity. Gelatinolytic activity was measured by a modification of the method of Hu et al. [3]. Briefly, this method uses rat-tail tendon collagen reduced with NaB^3H_4 as substrate. We found that the substrate at 37°C tended to denature and was thus susceptible to other proteases including our gelatin-specific protease(s). Thus, the assay performed at 37°C may be considered an assay for proteases including gelatinases. On the other hand, at 23°C this denaturation does not occur and thus the assay is still valid for true collagenases.

Typically, 0.5 ml reconstituted media equivalent to one dish (see above) was added to 0.5 M Tris-HCl, pH 7.6/0.005 M $CaCl_2$/0.02% NaN_3. Then 0.15 mg ^3H-labeled rat-tail tendon collagen (spec. act. $1.2 \cdot 10^5$ cpm/mg) in 0.15 ml of the above buffer was added. The mixture was incubated at 37°C overnight. After incubation, the mixture was centrifuged (2 000 × *g*) for 10 min and an aliquot of the supernatant counted in a liquid scintillation spectrometer. Enzyme activity is expressed as cpm released per 18 h at 37°C.

Trypsin pretreatment. Where required, media from the pulmonary macrophages were trypsin pretreated. Reconstituted media (0.5 ml) were incubated at 37°C with 10 μg bovine pancreatic trypsin for 10 min. To stop the reaction, 100 μg soya bean trypsin inhibitor were added and the mixture incubated at room temperature for 15 min before the addition of ^3H-labeled rat-tail tendon collagen substrate.

Collagenase assay. To evaluate the digestion of collagen to TC_A and TC_B pieces, soluble [^{14}C]proline-labelled collagen was prepared from chick calvaria [10]. The culture media were identical to that

described in the report, except that α,α'-dipyridyl was not included. After homogenization the calvaria were extracted with 0.5 M acetic acid (500 ml) containing 4 mg pepsin. Collagen was then precipitated by addition of 20% NaCl to make the final concentration 5% NaCl. The precipitate was dissolved in 0.01 M acetic acid and dialyzed against 0.02 M sodium phosphate buffer pH 7.4. The second precipitate was redissolved in 0.01 M acetic acid and dialyzed against 0.05 M Tris-HCl, pH 7.6/0.005 M $CaCl_2$/0.02% NaN_3. Specific activity of the [^{14}C]proline chick calvaria collagen was $1.2 \cdot 10^6$ cpm/mg.

Collagenase assays were carried out by adding 0.5 ml sample and 0.2 ml [^{14}C]proline chick calvaria collagen (5 000 cpm) in 0.05 M Tris-HCl, pH 7.6/0.005 M $CaCl_2$/0.02% NaN_3 and incubating at 25°C overnight. After incubation, samples were dialyzed against 0.01 M acetic acid and lyophilized. Sample buffer was added to the lyophilized sample and the sample was electrophoresed as described below.

Slab sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Polyacrylamide (7.5%) slab gels were prepared and electrophoresis was performed on a Studier-type apparatus (Bio-Rad) using the procedure of Laemmli [11]. The only modification of this system was that 2-mercaptoethanol was not included in the sample buffer. Slabs were stained with 1.25 g Coomassie blue in a solution of methanol/water/glacial acetic acid (25 : 25 : 4) and destained in water/10% methanol/7% glacial acetic acid.

Autoradiography. Autoradiograms were developed from slab gels by soaking the gels in Enhance (New England Nuclear), for 1 h at room temperature followed by water for 1 h. They were then dried on a gel dryer (Model 224, Bio-Rad) and exposed to Kodak X-Omat R X-ray film (Eastman Kodak) for appropriate times.

Elastase activity. Elastase activity of the media was assessed by using NaB^3H_4-reduced bovine ligamentum nuchae elastin as a substrate according to the method of Stone et al. [12]. The assay buffer used was 0.05 M Tris-HCl, pH 7.6/0.14 M NaCl/0.005 M $CaCl_2$/0.05% NaN_3. Incubation took place in a 37°C water bath overnight. Porcine pancreatic elastase was used as a reference standard.

Using the substrate *N*-succinyl L-alanyl-L-alanyl-L-alanine *p*-nitroanilide (Bachem Chemical) described by Bieth et al. [13] elastase activity was also assessed in the human pulmonary macrophage media and fractions. The assay was modified as follows: 0.005 M $CaCl_2$ was added to the incubation buffer 2 M Tris-HCl, pH 8.0, and the incubation was performed at 37°C for 18 h. An incubation mixture containing porcine pancreatic elastase was always run as a reference standard.

PZ peptidase activity. Assay with *p*-phenylazobenzyloxycarbonyl-(PZ)-L-Pro-L-Leu-Gly-L-Pro-D-Arg (Sigma Chemical) as a substrate was performed as described by Wunsch and Heidrich [14] except that the buffer used was 0.05 M Tris-HCl, pH 7.6/0.005 M $CaCl_2$/0.02% NaN_3. Incubation was done at 37°C overnight, after which the incubation mixtures were acidified and extracted with ethyl acetate. The absorbance of the reaction product PZ-Pro-Leu dissolved in ethyl acetate was read at 320 nm in a spectrophotometer. Bacterial collagenase was always included as a positive control.

Neutral protease activity. Neutral protease activity was measured using bovine methemoglobin as a substrate by a modification of the method of Anson [15]. Methemoglobin (Sigma Chemical) 2.0 mg and [methyl-^{14}C]methemoglobin (New England Nuclear) 8.0 μg were dissolved in 1.0 ml 0.05 M Tris-HCl, pH 7.6/0.005 M $CaCl_2$/0.02% NaN_3. The specific activity of [methyl-^{14}C]methemoglobin was 24.4 μCi/mg. Total incubation volume was 0.1 ml of which 0.05 ml was substrate and 0.05 ml contained the enzyme to be assayed. After an overnight incubation at 37°C, 0.1 ml of 10% ice-cold trichloroacetic acid was added and the mixtures were placed on ice for 10 min to allow precipitation. After preincubation, the mixtures were centrifuged at $3\,000 \times g$ for 10 min. The trichloroacetic acid soluble radioactivity was determined in a liquid scintillation spectrometer. Trypsin was included in each assay as a positive control. This assay is senstive to 5.0 ng trypsin.

Angiotensin-converting enzyme activity. The assay method of Cushman and Cheung [16] was used to determine the presence of angiotensin-converting enzyme in human macrophage media. This assay measures the release of hippuric acid from the substrate hippuryl-L-histidyl-L-leucine (Peninsula Laboratories, Inc.). The assay buffer was changed to 0.05 M Tris-HCl, pH 7.6/0.005 M $CaCl_2$/0.1 M NaCl. Fractions from the Sephadex G-200 column (see Materials and Methods) which gave significant activity

against ^{3}H-labeled rat-tail tendon collagen were assayed by this method. A specific inhibitor of angiotensin-converting enzyme: SQ 14 225 [17] was always included with a duplicate of each sample. The assay was incubated at 37°C overnight.

Gelatinase assays. The gelatinase activity of human macrophage media was assayed by two different methods. Calf skin collagen [18] was denatured by heating at 60°C for 10 min in a water bath. The resulting gelatin, adjusted to a concentration of 2.0 mg/ml, was incubated with 0.5 ml of the macrophage reconstituted media at room temperature for various incubation times. Total incubation volume was 0.6 ml. Incubation buffers was 0.05 M Tris-HCl, pH 7.6/ 0.005 M $CaCl_2$/0.02% NaN_3. To stop the reaction 30% trichloroacetic acid was added to the incubation making the final trichloroacetic acid concentration 15%. The trichloroacetic acid, itself, was extracted with ether [19] and the samples were lyophilized. The lyophilized samples were hydrolyzed in 6 N HCl at 110°C, overnight, and analyzed for trichloroacetic acid-soluble hydroxyproline using a Beckman Amino Acid Analyzer.

The second assay for gelatinase activity used the [^{14}C]proline chick calvaria collagen, prepared as described above, as substrate. Chick calvaria gelatin was made by heating the collagen to 60°C for 10 min. Incubation mixtures contained 0.5 ml sample and 0.3 ml gelatin in 0.05 M Tris-HCl, pH 7.6/0.005 M $CaCl_2$/ 0.02% NaN_3. Incubation was overnight at room temperature. Incubation mixtures were placed on a Bio-Gel A-1.5 (Bio-Rad) column, with dimensions 1.5 X 70 cm, according to the method of Piez [20]. Fractions of 3.0 ml were collected and their radioactivity was determined in a liquid scintillation spectrometer. $^{3}H_2O$ was used to determine the bed volume of the column.

Molecular sieve chromatography. Molecular sieve chromatography was performed on reconstituted human pulmonary macrophage media at 5°C, on a column with dimensions 1.5 X 100 cm containing Sephadex G-200 (Pharmacia Fine Chemicals) equilibrated with the buffer system 0.05 M Tris-HCl, pH 7.6/0.14 M NaCl/0.02% NaN_3. Flow rate of the column was 6 ml/h and 3 ml fractions were collected. The absorption of each fraction at 280 nm was determined. Appropriate standards were used to calibrate the column as previously described [21]. Fractions were analyzed individually for collagenolytic activity. Fractions before the bovine serum albumin peak were pooled and concentrated by ultrafiltration with an XM-50 membrane (Amicon) before analyzing for collagenolytic activity. Fractions after the albumin peak were pooled and concentrated by ultrafiltration with a PM-10 membrane (Amicon).

Results

Isolation of enzyme activity

Gelatinolytic activity was first measured against insoluble ^{3}H-labeled rat-tail tendon collagen using reconstituted media from cultured macrophages of volunteers. If the reconstituted medium is first treated with trypsin, the amount of gelatinolytic activity is increased 2.5–10.0-fold. There is also a wide variation in enzymatic activity among volunteers, although all volunteers were smokers and their cells were cultured in the same manner.

Before carrying out extensive characterization of the enzymatic activity, we removed the large amount of bovine serum albumin from the media (0.2%). This was accomplished by chromatographing the reconstituted media on a Sephadex G-200 column. Fractions before the albumin peak (pre-albumin) and after the albumin peak (post-albumin) were pooled and assayed for collagenolytic activity. A fraction within the albumin peak was also included. Table I represents the gelatinolytic activity found in these pools. Three patterns of activity were found. In sample 1 all the gelatinolytic activity was found in the post-albumin fractions. In sample 2 gelatinolytic activity was found only in the pre-albumin fractions. Finally, sample 3 showed activity in bith the pre- and post-albumin fractions. Gelatinolytic activity was never found in the albumin peak.

Besides removing albumin from the media, molecular sieve chromatography had another effect. After passage through the Sephadex G-200 column, the gelatinolytic activity recovered no longer required trypsin activation (data not shown).

Because of the presence of gelatinolytic activity in fractions that elute before bovine serum albumin, we hypothesized that the activity is present in at least two forms. Rechromatography of the pre-albumin fractions from pattern 2 or pattern 3 (Table I) on the Sephadex G-200 column appears to support this

TABLE I

ENZYME ACTIVITY AGAINST ^{3}H-LABELLED RAT-TAIL TENDON COLLAGEN OF SAMPLES BEFORE AND AFTER SEPHADEX G-200 CHROMATOGRAPHY

Albumin eluted at 124–154 ml. The number of batches for each sample was at least two. Samples were treated with trypsin (10 μg) followed by soybean trypsin inhibitor (100 μg) before assay. Pretreatment with trypsin before assay did not result in increase in enzymatic activity. Protein was measured spectrophotometrically using bovine serum albumin (BSA) as a standard. Pre-BSA, pre-albumin fraction, and Post-BSA, post-albumin fraction as obtained from Sephadex G-200 chromatography.

Sample	Enzyme activity (^{3}H CPM released/18 H per 100 μg protein)		
	Starting Material	Pre-BSA (Column Effluent 84–124 ml)	Post-BSA (Column Effluent 154–196 ml)
1	$1.1 \cdot 10^2$	0	$7.4 \cdot 10^3$
2	$3.1 \cdot 10^1$	$1.8 \cdot 10^3$	0
3	$2.8 \cdot 10^1$	$3.0 \cdot 10^3$	$3.5 \cdot 10^3$
2A	Pre-BSA Pool from Sample 2		
	$1.8 \cdot 10^3$	0	$4.1 \cdot 10^3$

conclusion. Table I contains these results: when active pre-albumin fractions were rechromatographed, the gelatinolytic activity disappeared from the pre-albumin fractions and was found only in the post-albumin fractions. In data not shown, further chromatography of the post-albumin fraction on Sephadex G-100, yielded enzyme activity in the post-albumin fractions. These results support, but do not prove, the hypothesis that the enzyme activity exists in more than one form. No further purification of the enzyme was undertaken.

Characterization of enzyme activity

Since the ^{3}H-labeled rat-tail tendon collagen substrate at 37°C is susceptible to other proteases besides gelatinase [3], we decided to determine if the gelatinolytic activity observed was due to a gelatinase. The reconstituted media were treated with enzyme inhibitors and their effect was observed on the gelatinolytic activity against the ^{3}H-labeled rat-tail tendon collagen substrate: Enzyme activity is inhibited by EDTA (5.0 mM) and cysteine (50.0 mM), which are typical inhibitors of mammalian collagenases [22]. In contrast the activity is not inhibited by elastase inhibitors *N*-acetyl-L-Ala-L-Ala-L-Pro-L-Ala-chloromethyl ketone (1.0 mM), *N*-Acetyl-L-Ala-L-Ala-L-Pro-L-Val-chloromethyl ketone (1.0 mM) or phenylmethylsulfonyl fluoride (0.5 mM). Neither is inhibition noted with the specific trypsin inhibitors: tosyllysine chloromethyl ketone (1.0 mM) or soya bean trypsin inhibitor (0.1 mg/ml). The pre- and post-albumin fractions from the Sephadex G-200 column showed the same inhibition profiles. We also found that the pre- and post-albumin fractions were inhibited by 5% fetal bovine serum (Grand Island Biological Co.), 100% inhibition, and dithiothreitol (0.01 M, 75% inhibition). These latter agents were not tested with the unchromatographed media.

Since the inhibition profile of the gelatinolytic activity corresponded to that of a mammalian collagenase we decided to determine if the activity observed was due to a mammalian collagenase. One of the more specific tests for a true mammalian collagenase is that it cleaves the α chains of a collagen molecule into three-quarter (TC_A) and one-quarter (TC_B) length fragments [22]. For this assay we used as substrate acid pepsin extracted [^{14}C]proline-labelled chick calvaria collagen. This substrate was incubated for 6 h at 25°C with the pre-albumin fraction described above or tadpole collagenase. The pre-albumin fraction used had total activity against ^{3}H-labeled rat-tail tendon collagen that was greater than or equivalent to the tadpole collagenase employed. The incubation mixtures were applied to SDS-polyacrylamide slab gels and electrophoresed. Autoradiograms of the slab gels were made and resulting patterns are shown in Fig. 1. The bands containing tadpole collagenase, Track 1 and 6, show the expected three-quarter and one-quarter length frag-

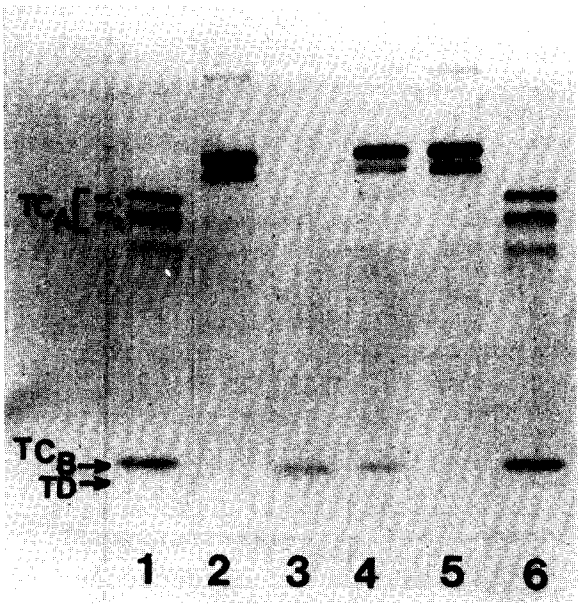

Fig. 1. Effect of gelatin-specific proteases on ^{14}C-labeled chick calvaria collagen, gelatin and native TC_A and TC_B fragments. [^{14}C]Gelatin was produced by heating [^{14}C]-collagen to 60°C for 10 min. TC_A and TC_B fragments were produced by incubating ^{14}C-labeled chick calvaria collagen (2900 cpm) with tadpole collagenase (10 μg) overnight at 25°C. Incubation buffer was 0.05 M Tris-HCl, pH 7.6/0.005 M $CaCl_2$/0.02% NaN_3. Pre-albumin pool from Sephadex G-200 chromatography (100 μl) was added to each substrate and incubated for 6 h at 25°C. The incubation mixtures were electrophoresed on an SDS-polyacrylamide slab gel. This figure shows an autoradiogram of the slab gel. Track 1, macrophage enzyme incubated with native TC_A and TC_B fragments; Track 2, macrophage enzyme incubated with collagen; Track 3, macrophage enzyme incubated with gelatin; Track 4, gelating alone; Track 5, collagen alone; Track 6, TC_A and TC_B fragments alone. TD is tracking dye.

ments. However, [^{14}C]proline-labelled collagen digestion products for the pre-albumin fraction are not seen in the autoradiogram (Track 2). Using longer incubation times (18 h at 25°C) for the pre-albumin fraction or the post-albumin fraction with ^{14}C-labeled chick calvaria collagen did not elicit any digestion fragments. Thus, the activity observed against ^{3}H-labeled rat-tail tendon collagen was apparently not due to a mammalian collagenase.

The effects of the human macrophage media enzyme after Sephadex G-200 chromatography on different protein and synthetic substrates was examined. These substrates included [^{3}H]elastin, [^{14}C]methemoglobulin, ^{14}C-labeled chick calvaria gelatin, Suc(Ala)$_3$-NA, hippuryl-L-histidyl-L-leucine, and PZ peptide (Table II). The enzyme appears active only against gelatin and inactive against all other protein or synthetic substrates tested. The results were identical whether pre- or post-albumin fractions were used.

The molecular size of the reaction products of gelatin digestion after treatment with the protease was examined. Gelatin with or without the protease treatment was chromatographed on a Bio-Gel 1.5-A column and the results are shown in Fig. 2. The ^{14}C-labeled radioactive reaction products are found in those fractions which eluted just before the elution of $^{3}H_2O$. The results were identical whether the pre- or post-albumin fractions were used. This pattern compares well with that of a gelatin-specific protease found in the media of cultures of rheumatoid synovium as described by Harris and Krane [5].

The digestion of calf skin gelatin was examined by monitoring the formation of trichloroacetic acid soluble hydroxyproline peptides (see Materials and Methods). The gelatin used contained 188 nmol hydroxyproline. A pre-albumin fraction which released 3 600 ^{3}H cpm/18 h in the gelatinolytic assay, described above, released 58 nmol trichloroacetic acid-soluble hydroxyproline. A post-albumin fraction which released 2 900 ^{3}H cpm/18 h released 44 nmol trichloroacetic acid-soluble hydroxyproline. There was considerable variation from preparation to preparation.

Finally, we examined the effect of the gelatin-specific protease on the three-quarter (TC_A) and one-quarter (TC_B) length fragments produced in collagen by tadpole collagenase. It is possible that this neutral protease might function in a concerted manner with tissue collagenases to degrade the collagen molecule. To produce native TC_A and TC_B fragments, tadpole collagenase was incubated with ^{14}C-labeled chick calvaria collagen overnight at 25°C. The gelatin-specific protease was then added to the incubation mixture, and incubation was allowed to proceed for an additional 6 h at 25°C. In addition, ^{14}C-labeled chick calvaria collagen or gelatin not pretreated with the tadpole collagenase was incubated with or without the specific protease under the same conditions.

TABLE II

ACTIVITY OF HUMAN MACROPHAGE MEDIA AND CONTROL ENZYMES ON DIFFERENT SUBSTRATES

Enzyme activity against ^{3}H-labeled rat-tail tendon collagen substrate was 4000 ^{3}H cpm/18 h at 37°C. All assays were incubated at 37°C overnight except the assay using gelatin as substrate which was incubated at 25°C overnight. Most incubations were carried out in 0.05 M Tris-HCl/0.005 M $CaCl_2$/0.02% NaN_3. TCA, trichloroacetic acid; Pre-BSA, pre-albumin fraction; Post-BSA, post-albumin fraction.

Substrate	Index measured	Enzyme(s) after G-200 chromatography		Trypsin (0.05 μg)	Bacterial collagenase (0.2 μg)	Pancreatic elastase (0.05 μg)	Rabbit lung homogenate (10 μg)
		pre-BSA	post-BSA				
^{14}C-labeled Chick Calveria Gelatin	TCA-Soluble Radioactivity, CPM	2800	2500	3300	–	–	–
[Methyl-^{14}C] Methemoglobin	TCA-Soluble Radioactivity, CPM	0	0	4700	–	–	–
NaB$^3H_4$-reduced elastin	Soluble Radioactivity, CPM	0	0	–	–	48 000	–
Suc-(Ala)$_3$-NA	$A_{410\ nm}$	0.000	0.000	–	–	1.256	–
PZ peptide	$A_{320\ nm}$	0.000	0.000	–	0.320	–	–
Hippuryl-L-histidyl-L-leucine	nmol hippuric acid/h per μg protein	0.02	0.007	–	–	–	30

The incubation mixtures were electrophoresed on an SDS-polyacrylamide slab gel and the developed autoradiogram in presented in Fig. 1. By comparing Track 1, gelatin specific protease incubated with TC_A and TC_B fragments and Track 6, TC_A and TC_B fragments alone, it can be seen that no apparent digestion has taken place. This is confirmed by the fact that no trichloroacetic acid-soluble ^{14}C-labeled radioactivity could be detected. However, by refering to Track 3 it can be seen that ^{14}C-labeled chick calvaria gelatin was completely digested during the same 6 h incubation. As expected ^{14}C-labeled chick calvaria collagen (Track 2) was undigested. These results suggest that the gelatin-specific protease does not digest undenatured TC_A and TC_B fragments under these conditions.

Discussion

This communication describes a gelatin-specific protease obtained from the media of cultured human pulmonary macrophages that is similar to other reported gelatinases [5,6]. It is active against gelatin, but inactive against collagen or the undenatured TC_A or TC_B fragments produced by the action of tadpole collagenase on native collagen. It is also inactive against other protein and synthetic substrates including [^{3}H]elastin, [^{14}C]methemoglobin, ^{14}C-labeled chick calvaria collagen, PZ peptide, Suc(Ala)$_3$NA and hippuryl-L-histidyl-L-leucine. Furthermore, the observed inhibition profiles were similar to that of other gelatinases, in that it is inhibited by EDTA, cysteine, dithiothreitol and fetal bovine serum [5,6].

Before Sephadex G-200 chromatography the enzyme is trypsin activatable. After gel filtration, the enzyme is active without trypsin pretreatment, suggesting the presence of a weakly bound inhibitor molecule in the original media. Other researchers have found that Sephadex chromatography also results in an active gelatinase not requiring trypsin pretreatment [24] and they have isolated a gelatinase inhibitor in their system.

Our chromatography results suggest that the enzyme activity exists in at least two molecular forms: one form eluting before and the other after albumin. Polymeric forms of gelatinase have not been described in the literature; however polymeric forms

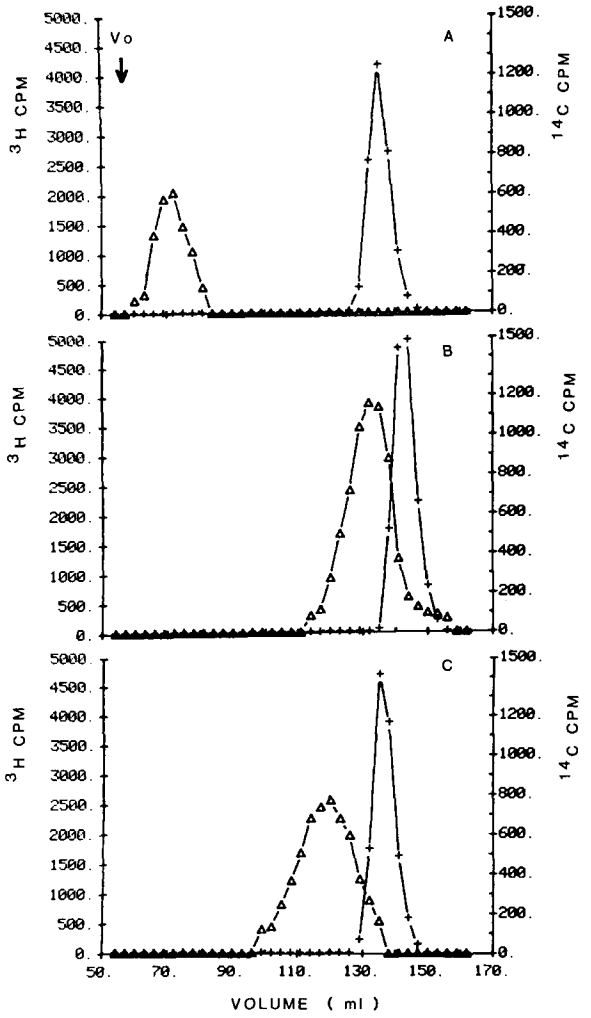

Fig 2. Patterns of elution of gelatin after various enzyme treatments from a column of Bio-Gel A-1.5 (1.5 X 70 cm). (A) [^{14}C]Calvaria gelatin alone, (B) [^{14}C]calvaria gelatin incubated with 0.5 μg trypsin and (C) [^{14}C]calvaria gelatin incubated with enzyme after Sephadex G-200 chromatography. Column bed volume determine with ^3H_2O. Column void volume determined with blue Dextran (△——△ ^{14}C cpm) (+——+ ^{3}H cpm). Incubation buffer 0.05 M Tris-HCl, pH 7.6/0.005 M $CaCl_2$/0.02% NaN_3. Incubation was overnight at 25°C.

of mammalian collagenases have been reported [25].

Other enzymes have been reported in human pulmonary alveolar macrophage culture media; a calcium-dependent activity against the elastase substrate $Suc(Ala)_3NA$ and a serine protease active against insoluble elastin [26,27]. Most of these activities are found intracellularly in the macrophages from smokers and non-smokers. However, a small amount is found extracellularly [26,27]. The enzyme activity reported here is not active against either elastase substrate nor does it have the inhibitor profile of either elastase-like enzyme. In a preliminary note several years ago, human pulmonary macrophages were reported to contain a true collagenase [28], however, no follow-up paper has appeared. On the other hand, pulmonary alveolar macrophages from rabbits have been shown to contain a true collagenase [29].

Our current studies do not indicate whether the gelatin-specific protease activity reported here is synthesized by the human pulmonary macrophages or has been previously endocytosed. A gelatin-specific protease has been reported in human polymorphonuclear leukocytes [6]. It was concluded that the insoluble elastin digesting activity in human pulmonary macrophages had its origin in human neutrophils [27]. Although our macrophage samples were almost neutrophil free (99% macrophages) when collected, they may have previously come in contact with neutrophils. The gelatin-specific protease found in human leukocytes [5] and the enzyme reported here share the following properties. Both enzymes are initially trypsin activatable before purification and require gelatin as their substrate. However, there are significant differences. When the leukocyte enzyme is chromatographed on Sephadex G-200, enzyme activity appears only in the pre-albumin fractions. No evidence of a lower molecular weight form of the enzyme is presented. Also the neutrophil enzyme activity is *p*-chloromercuribenzoate activatable (160% increase in activity) [6]. *p*-Chloromercuribenzoate does not activate the gelatinase described in this report.

The biological significance of gelatin-specific proteases is the subject of current discussion and investigation. As noted, vertebrate collagenases are capable of producing a single scission through the three chains of a native collagen molecule [22]. This results in a lowering of the melting temperature of the resulting collagen pieces [23]. True vertebrate collagenases are not capable of catalyzing the further degradation of these products (TC_A and TC_B) and neither is the gelatin-specific protease reported here. It is generally assumed that nonspecific enzymes

continue the degradation of the collagen pieces TC_A and TC_B after they, as a result of their lowered melting temperatures (T_m) spontaneously denature. Recently, researchers have discovered systems of enzymes from different tissues capable of acting at different sites in the degradation of collagen and other connective tissue. Sunada and Nagai [30] have described three gelatinases with different substrate affinities from embryonic chick skin explants. Sellers et al. [4] reported a specific collagenase, a gelatinase and a cartilage proteoglycan degrading enzyme from rabbit bone explants. The human polymorphonuclear leukocyte also contains several enzymes involved in collagen digestion: a collagenase [31], a gelatinase [6] and an enzyme which splits the crosslink region of collagen [32]. Although we have uncovered no other protease activity in our human macrophage cultures, the gelatinase reported here may participate with proteases from other tissue sources in the degradation of lung collagen. We have not ruled out the possibility that our enzyme preparations contain more than one gelatin-specific protease.

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