PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 168, 238–244 (1981)

An Elastase Inhibitor from Isolated Bovine Pulmonary Macrophages[1] (41267)

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Abstract. The interactions of a partially purified elastase inhibitor from bovine pulmonary macrophages were investigated using pancreatic elastase. The inhibitor was prepared from lysed pulmonary macrophages obtained by lavage of excised bovine lung lobes. In the presence of pancreatic elastase, the inhibitor formed a stable complex. Complex formation did not occur, however, if the elastase was first inactivated by pretreatment with phenylmethylsulfonyl fluoride. Fractions containing inhibitory activity were recovered from calibrated columns of Sephadex G-100 or G-150 in the molecular weight range of 40,000–50,000. Following incubation of the inhibitor with elastase, the inhibitor–elastase complex eluted in the molecular weight range of 65,000–75,000, suggesting a 1:1 molar ratio of inhibitor to elastase. The partially purified inhibitor did not inhibit trypsin, indicating that the bovine macrophage inhibitor did not arise from endocytosis of α-1-protease inhibitor.

A general hypothesis concerning the pathogenesis of pulmonary emphysema states that elastolytic proteases, occurring in amounts greater than the endogenous level of functional protease inhibitors, are responsible for the loss of lung elastic fibers and tissue recoil that are characteristic of emphysema (1–5). Elastolytic proteases from several sources have been implicated as possible mediators of lung damage (6–11). Attention has been directed primarily at the elastases arising from polymorphonuclear leukocytes (8) and pulmonary alveolar macrophages (PM) (10, 11).

There are numerous mechanisms for the introduction of elastases into lung tissues. For example, the cytoplasmic granules of neutrophils contain elastases effective in producing emphysematous lesions when released by cellular disruption (12) or phagocytosis (13). Similarly, macrophages have also been shown to be sources of elastases. Macrophages cultured *in vitro* can actively generate and secrete elastase after phagocytosis of latex microparticles (10, 11).

Furthermore, it has been demonstrated that purified leukocytic elastase binds rapidly and specifically to pulmonary macrophages *in vitro* and that the associated elastase is localized into phagolysosomes (14). One might postulate that the stored leukocytic elastase is eventually secreted from PM following appropriate stimulation.

Although leukocytic granule elastase may be readily detected, elastase activity from PM homogenates has either been below the limits of detection (7, 15) or observed in comparatively very low amounts. From the data of Hinman *et al.* (16) it may be estimated, for example, that the intracellular levels of elastase activity in pulmonary macrophages is approximately 2% of the amount present in neutrophils. Conceivably, the failure to detect elastase activity in PM extracts may be due to the presence of elastolytic inhibitors present in the lysate.

Indeed, the suggestion of PM-associated elastase inhibitors has been supported by several workers (17–19). For example, Blondin and Janoff (17) have described a potent inhibitor of the esterolytic activity of human neutrophil elastase from human PM

[1] This work was supported in part by a grant from the National Institute of Health, No. HL-18918 and a contract from the Electric Power Research Institute, No. RP-1639.2.

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0037-9727/81/100238-07$01.00/0

cytosol. Based upon its relative inhibitory activity toward a synthetic elastase substrate, the PM elastase inhibitor was noted to be several times more potent than a neutrophilic elastase inhibitor Janoff and Blondin (20) had described earlier. They have suggested that such inhibition may represent an additional determinant in the elastase-dependent component of pulmonary diseases, such as emphysema.

This study was initiated to determine whether bovine PM contain elastase inhibitors as determined by specific, elastase-sensitive substrates. Herein, we describe the partial isolation and characterization of an elastase inhibitor present in the soluble fraction of bovine PM lysates.

Materials and Methods. *Isolation of bovine alveolar macrophages.* For preparation of PM lysates, three to four bovine lung lobes were used. The procedure for PM isolation is similar to the technique described by Myrvik *et al.* (21). Briefly, cardiac lung lobes were obtained immediately after slaughter from healthy, young adult steers at a nearby abattoir. Lung lobes were kept on ice and lavaged within 1 hr after slaughter with ice-cold calcium- and magnesium-free phosphate-buffered saline (PBS). Lavage was performed by inserting a blunt, 15-gauge needle into a major bronchus. PBS (120 ml) was initially instilled to inflate the lobe and was followed by 7 successive cycles of injection and withdrawal of PBS (60 ml/cycle). Combined lavage fluid was centrifuged at 1200*g* for 30 min at 4° to separate cells. Red blood cell contamination was minimized by NaCl hypotonic–hypertonic lysis. Cells were recentrifuged and the supernatant was discarded. Isolated cells were then resuspended in 10 m*M* Tris–HCl buffer (pH 7.6) containing 20 m*M* NaCl, 0.5 m*M* $CaCl_2$, and 0.005% sodium azide. Aliquots were removed for assessment of viability (trypan blue dye exclusion), total cell yields (hemacytometer counting), and differential cell counts (Wright's stain) (22).

Preparation of PM lysates. Isolated PM were lysed by sonication (Virtis Cell Disrupter, Gardiner, N.Y.). The lysate was then centrifuged at 22,000*g* for 30 min at 4° and the supernatant was decanted and lyophilized. Total soluble protein was determined by the method of Lowry *et al.* (23) using bovine serum albumin (BSA) as a reference standard.

Estimation of elastase and trypsin inhibition. Inhibitory activity toward pancreatic elastase was measured using *N*-succinyl- L- alanyl- L- alanyl- L- alanyl-*p*- nitroanilide (SLAPN, Sigma Chemical Co., St. Louis, Mo.) and tritium labeled, insoluble bovine ligamentum elastin (Elastin Products, St. Louis, Mo.) substrates. SLAPN was chosen based on its stability and specificity under assay conditions; particulate elastin was selected to confirm inhibition observed with the synthetic substrate.

Stock solutions of SLAPN were prepared according to Bieth *et al.* (24) by making a 75 m*M* solution of SLAPN in *N*-methylpyrrolidone. Samples to be tested for elastase activity were buffered in 0.2 *M* Tris–HCl (pH 8.0) containing 5 m*M* $CaCl_2$. The substrate was tested against purified porcine pancreatic elastase (Elastin Products, St. Louis, Mo.) The release of *p*-nitroaniline was measured colorimetrically at 410 nm following 20 hr of incubation at 37°. In typical assays the inhibitor fractions were tested against elastase added at 1, 5 or 10 ng per sample. The final reaction mixture contained elastase, 0.5 mmole Tris–HCl (pH 8.0), 12 μmole $CaCl_2$, 5 μg BSA, 1.88 μmole SLAPN, and varying amounts of inhibitor in a total volume of 2.5 ml. For each set of assays, the release of *p*-nitroaniline was also estimated in reaction mixtures containing 1 to 10 ng of elastase without inhibitor to establish a standard curve.

In addition, elastase inhibition was measured as the release of radiolabeled peptides from an insoluble elastin substrate. The elastin was first chemically reduced with sodium [^{3}H]borohydride (ICN Radioisotopes Division, Irvine, Calif.). The reduction was performed as described by Takahashi *et al.* (25). The assay conditions were similar to those described for the SLAPN assay except the final volume was 0.4 ml and SLAPN was replaced by 0.4 mg of tritiated elastin (sp act, 330,000 dpm/mg). The reaction mixtures were incubated for 48 hr with gentle agitation in a shaking water bath at 37°. The release of tritiated peptides into the buffer was estimated fol-

lowing centrifugation at 9000*g* for 2 min (Beckman Microfuge, Palo Alto, Calif.) to separate the peptides from the insoluble substrate (26).

Estimates of tryptic activity were also performed using the synthetic substrate *p*-tosyl-L-arginine methylester hydrochloride (TAME, Sigma Chemical Company), Typically, up to 20 ng of trypsin (Sigma Chemical Company) was tested per assay. The composition of the reaction mixture and conditions for these assays have been described by Hummel (27) and were modified by extending the incubation period to 20 hr.

Partial purification of the bovine PM inhibitor. The PM lysate was concentrated using a negative pressure dialysis device (Pro-Di-Con-Mem, Bio-Molecular Dynamics, Beaverton, Ore.) and the bulk of the protein was separated from the inhibitor by gel filtration chromatography. The concentrated extract was applied to a 1.5 × 40-cm column of Sephadex G-100, equilibrated, and eluted with 0.2 *M* ammonium acetate (pH 7.6). Following gel filtration, fractions containing inhibitor were concentrated by lyophilization.

Elastase binding studies. To investigate the interaction between the inhibitor and elastase, experiments were performed similar to those described by Largman *et al.* (28), using radiolabeled enzyme. Iodine-125-labeled porcine pancreatic elastase was prepared with a commercial protein iodionating kit (Radioiodination System, New England Nuclear, Boston, Mass.). In typical experiments, crude, unconcentrated macrophage extracts or column fractions containing inhibitor were added to solutions (total volume 1 ml) containing labeled ^{125}I-elastase, 5 μg of albumin, 200 μmole NaCl, 50 μmole $CaCl_2$, and 100 μmole Tris (pH 7.6). Following incubation (1 hr at 25°), the solutions were applied to either a 1.5 × 40-cm column of Sephadex G-100 or 1 × 50-cm column of Sephadex G-150 equilibrated in 0.1 *M* Tris (pH 7.6) buffer containing 0.2 *M* NaCl.

The binding of elastase to components in fetal calf serum was also investigated in a similar manner. In these experiments, the labeled elastase was reacted with 100-fold molar excess phenylmethylsulfonyl fluoride (PMSF, Sigma Chemical Company) prior to addition of the inhibitor (27) to determine the effect of elastase inactivation on macrophage inhibitor binding.

Results. *Macrophage recovery and inhibitory activity.* Saline lavage of bovine lung lobes provided large quantities of PM. Normally, 100 × 10^6 PM could be obtained from the pooled lavage of three lung lobes, resulting in approximately 5 mg of solubilized protein. The cellular composition of bovine lung lavage fluid was almost exclusively PM (90%) with lymphocytes (less than 8%) and neutrophils (less than 2%) as rare contaminants. Viability usually exceeded 80%. When crude PM extracts were tested for elastase inhibition against 10 ng of pancreatic elastase using the SLAPN substrate, approximately 25 μg of the extract effected a 50% reduction in elastase activity (Fig. 1).

Partial purification and characterization of the inhibitor. Approximately 50% of the initial inhibitory activity was recovered after concentration and gel filtration (Fig. 1). Following gel filtration, the majority of the inhibitory activity was recovered in the pooled column fraction 3, corresponding to a molecular weight of 40,000–50,000. Compared to the crude extract, the specific inhibitory capacity per milligram of protein was increased 5- to 10-fold in fraction 3.

When fraction 3 was incubated with labeled elastase, the elution profile of radioactivity as ^{125}I-labeled elastase was shifted from that of pancreatic elastase to a component corresponding to molecular weight 65,000–75,000 (Figs. 2 and 3). Similar results were obtained when the crude extract was used without partial purification (Fig. 2). In the presence of fetal calf serum, however, the ^{125}I-labeled elastase migrated as high-molecular-weight components at the void volume.

That the macrophage inhibitor interacts directly with elastase could also be demonstrated, in part, by the observation that prior inactivation of elastase by phenylmethylsulfonyl fluoride appeared to interfere with the binding of elastase to the inhibitor (Figure 3).

Finally, the PM inhibitor was not active against trypsin. Although near complete inhibition of elastase (10 ng) was observed

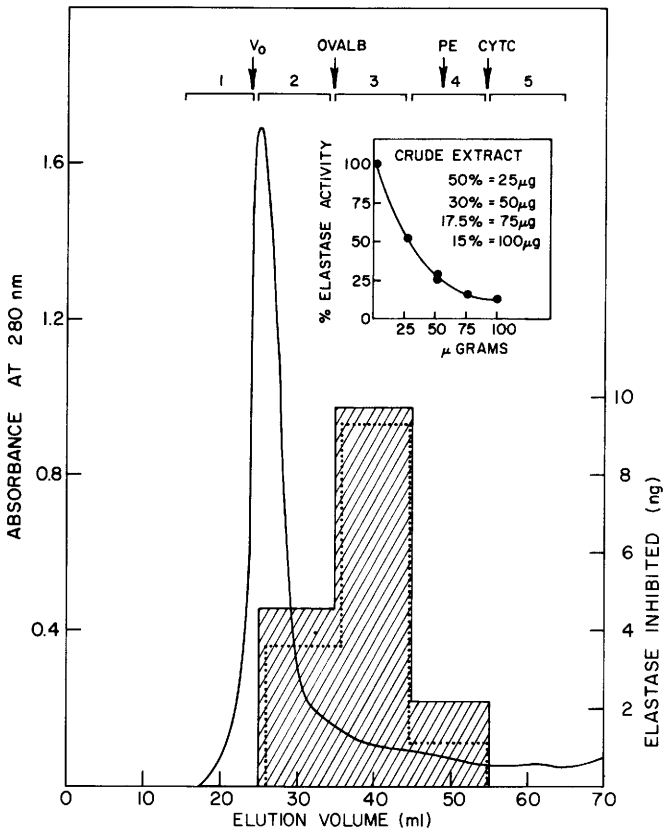

FIG. 1. Fractionation of concentrated bovine macrophage lysate on a 1.5 × 40-cm column of Sephadex G-100. The eluant (0.2 *M* ammonium acetate, pH 7.6) was divided into five fractions corresponding to approximately 10 ml each. Each fraction was concentrated to 1 ml and 20 μl was assayed for elastase inhibitory activity using SLAPN or tritiated elastin as substrates. The majority of inhibitory activity was observed in fraction 3 and corresponded to 40,000–50,000 mw proteins. The small insert shows the average elastase inhibitory capacity of two crude macrophage extracts when tested using SLAPN as substrate (see text). The arrows at the tope of figure correspond to column void volume and the elution volumes for ovalbumin, porcine pancreatic elastase and cytochrome *c*.

when 75.0 μg or more of the crude macrophage extract was added to the assays, inhibition of tryptic activity using the TAME substrate was not observed.

Discussion. Considerable attention has been directed at the role of PM in pulmonary diseases. Of particular interest is the ability of macrophages to secrete or internalize elastase (7, 10, 14). However, only limited data are available on the elastolytic inhibitory capacity of macrophages (17). To our knowledge, studies employing PM from freshly excised bovine lung lobes have not been reported in the literature. Bovine PM have, however, been used extensively in this laboratory because they are a reliable source of macrophages; simply and inexpensively retrieved in large numbers. Also, based upon the functional parameters of macrophage attachment and phagocytosis kinetics, bovine macrophages behave similarly to human macrophages in culture (unpublished observations). Clearly, the existence of a readily obtained source of PM will help expedite the study of basic PM biochemistry and physiology.

In this preliminary report, we have shown that lysates of freshly harvested

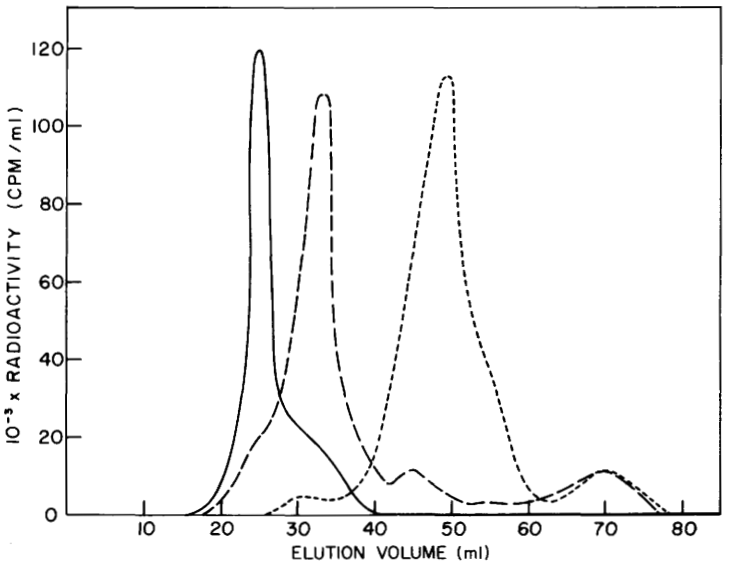

FIG. 2. Elution profiles for ^{125}I-pancreatic elastase chromatographed alone (. . .) and following incubation with either 3000 μg of protein from the crude macrophage extract (– – –) or 4500 μg of fetal calf serum (——). The complex formed by reaction of elastase with components in the macrophage extract eluted similarly to proteins with molecular weights of 65,000–75,000.

bovine PM are capable of inhibiting elastase. The gel filtration studies indicate that the inhibitor binds to pancreatic elastase, possibly forming complexes in 1:1 molar ratios. For example, the inhibitor fraction which corresponds to proteins with an apparent molecular weight of 40,000–50,000, produced an apparent 65,000–75,000 MW complex in the presence of pancreatic elastase (molecular weight 25,000). Furthermore, binding did not occur following inactivation of elastase by preincubation with PMSF (also cf. Ref. (28)).

The human PM inhibitor studied by Blondin *et al.* (17), was shown to be effective in the inhibition of human leukocytic granule esterase using the synthetic elastase substrate, tertiary butyloxycarbonyl-L-alanine-*p*-nitrophenol (*t*-BOC). When compared to a similar elastase inhibitor in leukocyte homogenates described in an earlier paper (20), the PM inhibitor was estimated to be approximately four times more potent than the leukocytic inhibitor. In this study, bovine PM homogenates were shown to inhibit pancreatic elastase based on measurements using specific synthetic and natural substrates. Due to the questionable specificity of t-BOC as an elastase substrate (15), direct comparison between the efficiencies of the bovine and human PM homogenates as elastase inhibitors is not possible.

Levine *et al.* (15) has reported that PM lysates from guinea pigs, hamsters, rabbits, and rats will inhibit human leukocytic granule extract elastase. However, these PM lysates do not inhibit pancreatic elastase. The apparent discrepancy in inhibitor behavior between rodent and bovine PM may reflect species differences in the specificity of affinity of the inhibitor elaborated by PM toward pancreatic or leukocytic elastases. In this regard, preliminary experiments by Hinman *et al.* (16) have shown that human PM lysates inhibit purified pancreatic elastase using SLAPN, suggesting that important species differences in inhibitor specificity may exist between humans and rodents. Alternatively, the discrepancy may be related to the techniques employed in lysate preparation, absolute inhibitor concentrations, or the different incubation times chosen for the

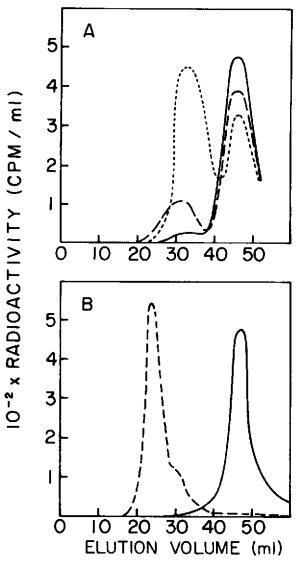

FIG. 3. Elution profiles for ^{125}I-elastase and complexes formed after reaction with partially purified fractions of the bovine macrophage inhibitor. In A. a quantity of inhibitor sufficient to inhibit 5 ng of elastase was incubated with 5 ng of ^{125}I-elastase. Following a 1-hr incubation at 25° the reaction mixture was chromatographed on a 1 × 50-cm column of Sephadex G-150. The majority of the radioactivity eluted as a 65,000–75,000 MW complex (...). Complex formation was significantly reduced when PMSF-treated elastase was used (– – –). For comparison, the elution of noncomplexed ^{125}I-elastase is also shown (——). In B, the elution of elastase with (– – –) and without (——) preincubation in fetal calf serum is shown for reference.

elastase inhibition assay. In this study, *p*-nitroaniline release was monitored after 20 hr rather than the 20-min period incorporated in the studies of Levine *et al.*

Evidence that the bovine inhibitor does not represent internalized serum inhibitors comes from two observations. First, serum contains large quantities of both elastase and trypsin inhibitors. Representative inhibitors in human serum include α-2-macroglobulin (α-2-M, approximately 725,000 daltons) and α-1-protease inhibitor (α-1-Pi, approximately 55,000 daltons). Both α-2-M and α-1-Pi strongly inhibit trypsin, yet we were unable to demonstrate inhibition of trypsin with bovine PM homogenates. Also, the bovine elastase inhibitor is unlike the bovine serum inhibitor of elastase and trypsin which was reported by Wu and Laskowski (29). They found that the bovine serum inhibitor has a molecular weight of 71,000; considerably greater than that observed for the bovine PM inhibitor (40,000–50,000 daltons). We did not determine if the bovine inhibitor was similar to the human bronchial inhibitors described by Ohlsson and Tegner (30). While these observations suggest that the bovine PM elastase inhibitor is unique to PM and may not be the result of PM endocytosis of serum inhibitors, further studies are needed to determine the origin of the bovine PM inhibitor.

It may be postulated that the binding of pancreatic elastase to the inhibitor occurs through the active site, serine residue of elastase, because complex formation was markedly reduced when elastase was preincubated with PMSF. Largman *et al.* (28) have reported similar behavior of α-1-Pi and human pro-elastase.

Distinctive forms of elastase have been described in the literature. Depending upon the species, each elastase exhibits different substrate specificities, pH optima, and inhibitor sensitivities (10, 11, 16). Notably, human PM have been shown to secrete a metal-dependent elastase-like enzyme using the SLAPN substrate. However, metal-dependent elastase activity has not been observed with the insoluble elastin substrate in human PM. Conversely, murine peritoneal macrophages generate a metalloprotease when tested against tritiated elastin, but not against SLAPN (16). These are important differences when attempting to compare elastases and inhibitor interactions from different species.

We have recently observed that, *in vitro*, nonstimulated bovine PM secrete small amounts of a metal-dependent elastase into the culture medium if tested with a tritiated elastin substrate (unpublished data). For example, 100 million bovine PM will secrete from 0.2 to 0.4 μg of elastase (expressed as pancreatic elastase equivalents) during the first 2 days of culture. In comparison, the amount of inhibitor within 100 million PM results in 4–5 mg of soluble protein; an amount sufficient to inhibit approximately 0.5–1.0 μg of pancreatic elastase. Based on these observations, it ap-

pears that the PM lysate contains a twofold excess of inhibitor relative to the amount of elastase normally secreted over a 2-day culture period (assuming the bovine PM pancreatic elastase inhibitor also inhibits bovine PM elastase). Although it is not clear how intracellular protease inhibitors are involved in tissue homeostasis, it is conceivable that alterations of intracellular inhibitor levels may upset the local protease-inhibitor balance.

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Received March 6, 1981, P.S.E.B.M. 1981, Vol. 168.