Connective Tissue Research

ISSN: 0300-8207 (Print) 1607-8438 (Online) Journal homepage: https://www.tandfonline.com/loi/icts20

Elastolysis of Insoluble Elastin

Mark Schuyler, Adi Gerblich, Laurence Peterson & Gregory Urda

Connective Tissue Research, 1984, Vol. 12, pp. 87–95
0300-8207/84/1202–0087/$18.50/0

ELASTOLYSIS OF INSOLUBLE ELASTIN†

MARK SCHUYLER,‡ ADI GERBLICH, LAURENCE PETERSON, and GREGORY URDA

Department of Medicine, Cleveland VA Medical Center and Case Western Reserve University, Cleveland, Ohio

(Received April 5, 1983; in final form June 13, 1983)

We developed an assay for measurement of elastolytic activity using insoluble ^{3}H-labelled particulate elastin adherent to plastic that is capable of detecting 150 picograms of pancreatic elastase. This equals or exceeds the sensitivity of the most sensitive previously reported systems, without requiring sodium dodecyl sulfate treatment of the elastin. Elastin digestion is dependent upon substrate and elastase concentration, but is not linearly related to time. This is partially attributable to elastase denaturation or autolysis under the assay conditions.

The assay could easily detect elastase secreted by either peritoneal or alveolar macrophages. Compared to previously described assays using substrates that closely resemble the physiologic substrate, this represents a considerable increase of sensitivity of detection of elastolytic activity of enzymes.

INTRODUCTION

Elastase, a neutral protease secreted by pancreatic acinar cells, polymorphonuclear leukocytes, macrophages and certain transformed cells, is thought to be important in the pathogenesis of pancreatitis,[1] arthritis,[2] atherosclerosis[3] and emphysema.[4] A variety of methods have been developed to measure this enzyme. The most sensitive methods to date (sensitivity 100 to 2000 picograms pancreatic elastase) use elastin fragments,[5,6] sodium dodecyl sulfate (SDS) treated elastin,[7–9] or do not distinguish between functional and nonfunctional enzyme.[11] Methods which utilize native elastin cannot detect less than 20 μg of elastase.[12–14]

In this report, we describe an elastase assay using labelled insoluble elastin, with very high sensitivity. We modified previous assays which utilize tritiated insoluble elastin by attaching elastin particles to plastic walls of microtiter wells and filling the wells with elastase containing solutions. This increased the substrate available to the enzyme so that we can reproducibly detect 150 picograms of porcine pancreatic elastase. This is not only more sensitive than previous systems using non-SDS treated elastin as substrate, but it also can readily detect macrophage (both alveolar and peritoneal) elastase.

† A preliminary report of this data appeared in Clin Res 30, 437A, 1982

‡ To whom correspondence should be addressed at: Cleveland VA Medical Center, 10701 East Blvd. Cleveland, Ohio 44106.

MATERIALS AND METHODS

Elastin

Bovine neck ligament elastin (200–400 mesh, No. E60, Elastin Products Company, St. Louis, MO) was labelled by the tritiated sodium borohydride reduction method by Amersham Corp., Arlington Heights, IL, using procedure TR5/74.[7,8] Specific activity of the elastin was 700 μCi/mg. This method labels aldehydes and reducible crosslinks. The elastin was suspended at 6 mg/ml in water and sonicated for 9 min. (Heat Systems, Ultrasonic Inc., Plainview, NY) and stored at 4 °C. Prior to each assay, 50 μl of the above stock suspension (300 μg) was transferred to 0.05 M carbonate buffer, pH 9.6, sonicated again and adjusted to 14 μg/ml (9.9 μCi/ml) in 0.05 M carbonate buffer. Twenty five percent of the radioactivity of this elastin suspension is associated with particles and can be sedimented by centrifugation (100,000 × g, 30 min.). Non-particulate label is 10% precipitable with 5% trichloroacetic acid (TCA). Therefore, 25% of the total counts are particulate, 7.5% soluble and TCA precipitable and 67.5% soluble and not precipitated by TCA.

Elastase Assay

One hundred μl of the continuously stirred elastin suspension (1.4 μg, 0.99 μCi) was placed into each well of a 96-well flat-bottom microtiter plate (Costar No, 3596, Cambridge, MA). The microtiter plate was incubated 18 h at 4 °C in a humidified atmosphere to allow binding of the elastin particles to the plastic. The plate was then washed three times with 0.15 M phosphate buffered saline (PBS), pH 7.2 with 0.05% Tween-20 and 0.02% sodium azide (Sigma Chemical Corp., St. Louis, MO) by careful immersion and inversion of the plates in PBS-Tween. The total amount of elastin adhering to the plate was determined by the amount of radioactivity released into the supernatant by excess quantities of elastase.

Porcine pancreatic elastase (76 to 95 units/mg), and chymotrypsin (42 units/mg), were obtained from Sigma Chemical Corp., St. Louis, MO; 1-chloro-4-phenyl-3-L-tosylaridobutan-2-one ("TPCK")-treated trypsin (200 units/mg) from Worthington, Freehold, NJ; trypsin from Gibco, Grand Island, NY; collagenase (2,800 units/mg) from Advance Biofactures Corp., Lynbrook, NY; and pepsin (2500 units/mg) from Calbiochem, Palo Alto, CA. All enzymes were dissolved in 0.1 M Tris, pH 7.8 buffer. Each experiment consisted of a standard curve of dilutions of one lot of pancreatic elastase and unknown samples. One hundred fifty μl of the following final concentrations of pancreatic elastase (10^2, 10^3, 2.5×10^3, 5×10^3, 10^4, 2.5×10^4, 10^5, 10^6 picograms/ml) or unknown samples were added to quadruplicate wells of the microtiter plate.

After further incubation at 37 °C in a humidified atmosphere, the plates were harvested by withdrawing 10 μl from each well of the microtiter plate. Each sample was added to 2.5 ml Aquasol II (New England Nuclear, Boston, MA) and counted on a Packard Tricarb liquid scintillation counter. The mean results of quadruplicate wells are expressed as disintegrations per minute (DPM).

Macrophages

Activated rabbit alveolar macrophages, thioglycollate induced mouse peritoneal macrophages and conditioned media (CM) from macrophage cultures were obtained by previously described methods.[15]

RESULTS

Binding

The amount of elastin that remained bound to the wells after 18 h incubation during different experiments varied from 12% to 42%, but each well in a particular plate (one experiment) contained similar quantities of elastin, so that the coefficient of variation among quadruplicate replications was less than 15%. Many elastin particles were visible adhering to the side walls and bottom of the wells. To determine if we were measuring degradation of particulate elastin, or soluble elastin that was absorbed to the plastic walls of the wells, we added soluble elastin (i.e. that portion of the particulate substrate not sedimented by 100,000 × g, 30 min, which included 75% of label) to some plates, and compared the results with those obtained using uncentrifuged particulate elastin, treating both plates identically. Large amounts of elastase (150,000 picograms) released only 8% of the radioactivity from bound soluble elastin that was released from particulate elastin (Table I), despite the presence of 75% of the initial label in the soluble fraction. These results are compatible with either lack of absorption of soluble elastin to the wells, or lack of degradation of absorbed soluble elastin by pancreatic elastase. Although we cannot distinguish between these possibilities, data indicate that we are measuring degradation of particulate and not of soluble elastin, since most of the label released originates from the particulate rather than the soluble portion of our substrate.

To examine the relationship between the amount of ^3H released and the amount of elastin substrate present in the wells, 0.03 to 2.2 μg elastin were incubated with varying amounts of pancreatic elastase. As indicated in Figure 1, ^3H release was directly dependent upon

TABLE I

Enzymatic release of label from soluble vs particulate elastin substrate
Pancreatic elastase (picograms/well)

	150,000	1500	150	15	0
Particulate[†]	14,295[§] ±1416	11,818[§] ±836	3902[§] ±322	2342[§] ±308	1983[§] ±351
Soluble[‡]	1176[§] ±284	ND[‖]	ND[‖]	ND[‖]	748[§] ±144

[†]Uncentrifuged elastin substrate.
[‡]Equal amount of substrate centrifuged 100,000 × g, 30 min, Supernatant added to wells.
[§]Results are the arithmetic mean of three experiments, DPM ± SEM of quadruplicate wells label released after 18 h incubation at 37°C in a humidifed atmosphere.
[‖]Not determined.

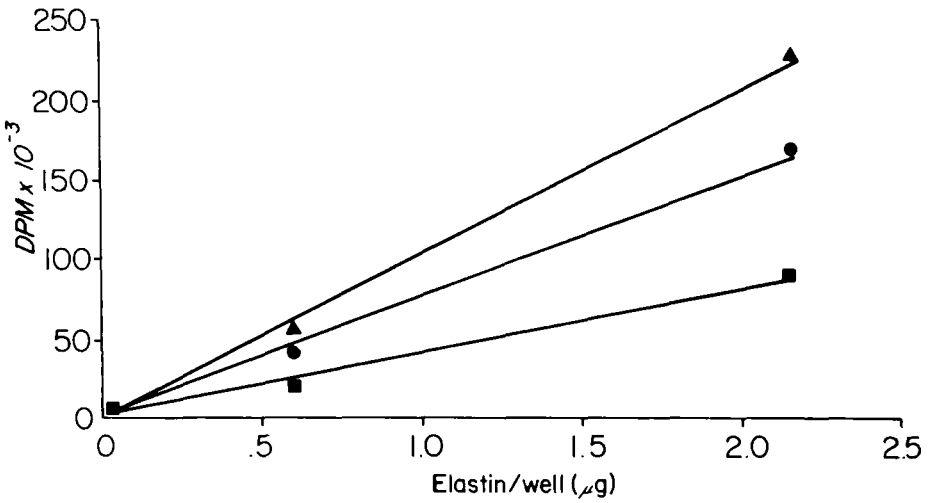

FIGURE 1 Substrate concentration vs ^3H release after 18 h incubation. Mean of three experiments: ▲ 150,000 picograms pancreatic elastase/well; ● 15,000 picograms pancreatic elastase/well; ■ 7,500 picograms pancreatic elastase/well.

the quantity of both enzyme and substrate in the wells. It is also apparent that larger amounts of pancreatic elastase could release more ^3H than smaller amounts, independent of the quantity of substrate.

Dose-Response and Kinetics

As indicated by Figure 2, the release of label from a constant amount of elastin substrate (0.6 μg/well) was dependent on both time and elastase concentration. Maximal release occurred with 15,000 picograms pancreatic elastase/well after 4 h, with 3750 picograms after 18 h and with 750 picograms after 96 h incubation. Note that each well received 0.15 ml of the elastase solution.

It appeared from the results in Figure 2 that the amount of label released was dependent upon time. However, ^3H release produced by large amounts of elastase was not linearly related to time. As illustrated by Figure 3, 1.5×10^6 picograms of elastase rapidly caused release of large amounts of ^3H, so that no more label was released after 4 h. In contrast, 1500 picograms elastase produced label release that was directly proportional to time over 18 h. However, 1500 picograms elastase did not induce ^3H release proportional to time over 96 h incubation as the ^3H release reached a plateau before 96 h (data not shown). To examine possible reasons for this phenomenon, we allowed pancreatic elastase to remain at 37 °C in a humidified atmosphere in microtiter plates without elastin for varying periods of time and then removed the enzyme and tested elastolytic activity using elastin coated microtiter wells. As indicated by Figure 4, pancreatic elastase lost activity over time under these conditions. Thus, enzyme denaturation or autolysis could contribute to the observed non-linear relationship between time and label release (Figures 2 and 3).

Therefore, we chose 18 h, as our standard incubation period because there was relatively little denaturation of elastase during this time period, yet considerable sensitivity (i.e. 150 picograms pancreatic elastase was consistently detectable). There was a linear relationship between the quantity of elastase and label release from 375 to 3750 picograms/well.

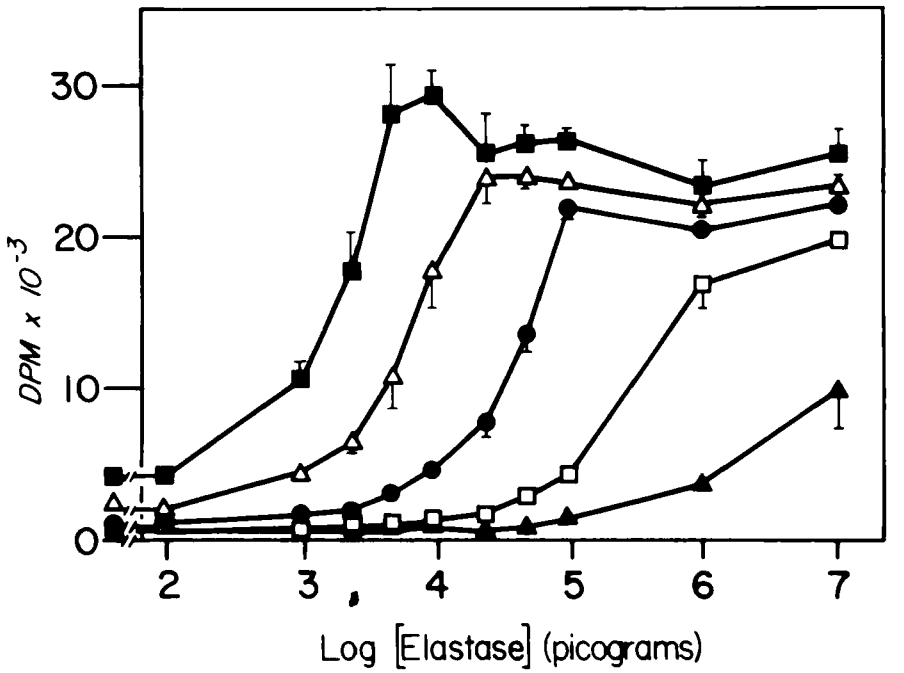

FIGURE 2 Kinetics of elastin degradation. ^3H release from 0.6 μg elastin/well after incubation. Mean and standard deviation of three experiments: ■ 96 h; △ 18 h; ● 4 h; □ 1 h; ▲ 15 min.

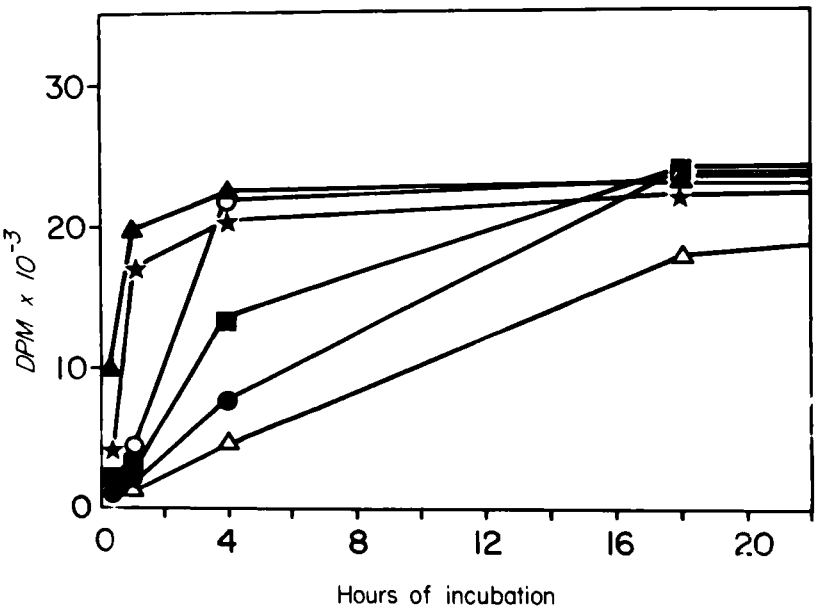

FIGURE 3 Cumulative release of ^3H using varying amounts of pancreatic elastase after 18 h incubation. Mean of three experiments: ▲ 1.5×10^6 picograms pancreatic elastase/well; ★ 1.5×10^5 picograms pancreatic elastase/well; ○ 1.5×10^4 picograms pancreatic elastase/well; ■ 7.5×10^3 picograms pancreatic elastase/well; ● 3.75×10^3 picograms pancreatic elastase/well; △ 1.5×10^3picograms pancreatic elastase/well.

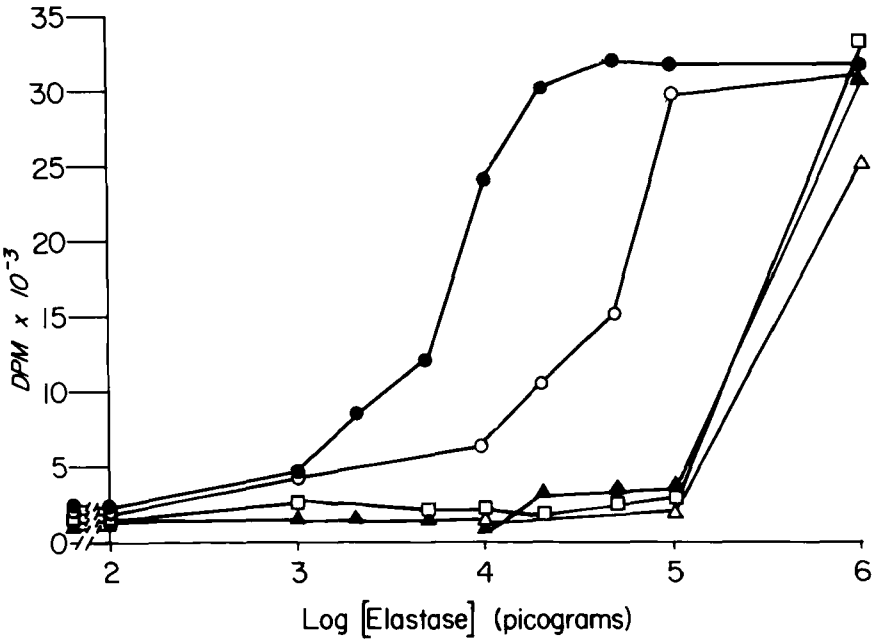

FIGURE 4 Stability of pancreatic elastase during 37°C incubation. Enzyme was incubated in microtiter wells without elastin substrate for varying periods of time, when aliquots were removed and tested for elastolytic activity. Mean of three experiments: △ 96 h incubation; ▲ 72 h incubation; □ 48 h incubation; ○ 18 h incubation; ● 0 h incubation.

We next investigated the nature of the degradation products that we detected in well supernatants by determining the percent of release ^{3}H that precipitated with 5% TCA. We found that 64% ± 2% (mean ± SEM of 3 experiments) of the label released by treatment with 150,000 picograms pancreatic elastase was TCA precipitable. This suggests that most of the ^{3}H released from bound elastin appears in the form of soluble polypeptide fragments of elastin and does not represent free ^{3}H release.

The ability of enzymes other than elastase to degrade the ^{3}H elastin is presented in Table II. The data indicate that collagenase and pepsin had no detectable elastolytic activity, whereas trypsin and chymotrypsin could cause label release. This is consistent with the known elastolytic activities of these enzymes. TPCK treated trypsin exhibited considerably less activity (6% of total available radioactivity), indicating that much of the trypsin elastolytic activity is due to chymotrypsin contamination.[15]

Elastolytic activity detected by this assay could be expressed as picograms pancreatic elastase, or as units defined by elastin degrading ability determined by using a different assay (i.e. manufacturer's units), or as the amount of elastolytic activity as defined in this report. We could determine the amount of elastin that adhered to the wells (maximum released label). Since elastin degradation is not linearly related to time, we defined our enzyme units in terms of picograms elastin degraded per 18 h. This allows for variation of elastin binding to the plastic wells between different experiments. The calculation is as follows:

TABLE II

Comparison of Elastolytic Activities of Various Enzymes

Enzyme	150,000†	15,000†	7500†	3750†	1500†	150†
Pancreatic elastase	30,650‡	32,250‡	31,460‡	16,240‡	3160‡	3160‡
Collagenase	0‡	ND	ND	0‡	ND	ND
Pepsin	20‡	ND	ND	290‡	ND	ND
Chymotrypsin	4,340‡	ND	ND	290‡	ND	ND
Trypsin	31,860‡	18,830‡	ND	1790‡	1580‡	ND
TPCK-treated trypsin	2060‡	ND	ND	ND	950‡	ND

ND: Not determined

†Enzyme concentration (picograms/well)

‡Results are the arithmetic mean of three experiments. DPM released from quadruplicate wells after 18 h incubation.

$$\text{Picograms bound elastin} = \frac{\text{Maximum DPM released/well}}{2.22 \times 10^6\ (700)}$$

2.22×10^6 DPM/mμCi. 700 is the specific activity of the elastin substrate (μCi/mg)

For each experiment, we plotted a standard curve with different amounts of pancreatic elastase and defined the amount of elastase that produced 50% of the maximal DPM released as 5×10^5 microunits. The results of other samples were expressed as microunits by relating DPM produced by the sample to the standard curve.

We next examined the ability of this assay to detect other elastolytic enzymes. We chose macrophage elastase derived from induced mouse peritoneal exudate macrophage and activated rabbit alveolar macrophages since both of these cell types have previously been demonstrated to secrete elastase.[14,17–21] Conditioned media (CM) from 10 of 16 activated rabbit alveolar macrophage cultures exhibited elastolytic activity (mean activity 6.6×10^5 microunits), as did CM from mouse thioglycollate induced peritoneal macrophages (mean activity 2.4×10^5 microunits). CM from cultures without cells did not demonstrate activity.

DISCUSSION

Previously described assays measuring elastase activity have either been relatively insensitive,[7,12,14,20] unable to distinguish active from inactive enzyme,[10,11] or measured processes other than elastolysis[22,23] which are not related to tissue destructive activity.[4] We have developed an improved assay using insoluble elastin which can detect as little as 150 picograms pancreatic elastase. The elastolytic activity represents dissolution of insoluble particulate rather than soluble elastin, and uses a substrate that resembles elastin in tissues such as arterial walls and the lung more closely than SDS-treated elastin. Using this assay, we can readily detect elastolytic activity secreted by macrophages and thus can apply this technique to enzymes other than pancreatic elastase. Release of label in this assay is dependent upon substrate and elastase concentration, but is nonlinear with respect to time. This was at least partially caused by denaturation or autolysis of enzyme under our assay

conditions, but could also be secondary to the heterogenous nature of our particulate substrate. Since our substrate consists of different size particles adhering to plastic, the rate of label release may vary with time. It would be expected that smaller particles with a larger surface area/weight ratio would release label rapidly early and that larger particles would release label more slowly later in the incubation period.

This assay is more sensitive than radioimmunoassays. For example, Geokas *et al.*[10] could detect approximately 900 picograms of pancreatic elastase, but could not discriminate between functional and nonfunctional elastase, as radioimmunoassays detect antigenic determinants and not activity. Another sensitive system which measures label release from elastin fragments (alpha-elastin) attached to Sepharose does not use native elastin but can detect 100 picograms pancreatic elastase.[6] Similar limitations in elastase detection have been reported by Beiger and Scheele using soluble elastin.[5] Other assay systems have used insoluble elastin, which more closely resembles native elastin than soluble elastin, but these assays are much less sensitive than the radioimmunoassay described. For example, insoluble elastin impregnated in agar can only detect 20,000 picograms or more elastase activity.[4] Werb and Gordon[14] developed an assay system using labelled insoluble elastin which is limited to 5000 to 20,000 or more picograms of elastase. Subsequent modifications were able to improve sensitivity 5-fold by the addition of SDS.[7] The mechanisms of this increased sensitivity are unclear, but involve dissociation of enzyme-inhibitor complexes and perhaps conformational changes of the substrate.[17] Stone *et al.*[9] could detect 300 picograms of elastase using 7-day incubation periods and SDS-treated insoluble elastin. SDS treated elastin, compared to non-SDS-treated elastin, is more susceptible to degradation by proteolytic enzymes other than elastase.[18] Excess SDS depresses elastolysis[7,8] and SDS-treated elastin does not correspond to elastin *in vivo*. In addition, it has recently been demonstrated that the ability of macrophage elastase to degrade connective tissue matrix corresponds to its activity as determined with non-SDS-treated, rather than SDS-treated elastin.[24]

The assay system described in this report uses non-SDS-treated, insoluble elastin and is extremely sensitive. The increased sensitivity of our assay, compared to previous assays using radiolabelled non-SDS-treated insoluble elastin, is probably due to the use of very small amounts of elastin (1.4 μg vs 1 to 5 mg) and high specific activity of the elastin (700 μCi/mg vs 0.2 to 0.3 μCi/mg.[14,17,21,24] This method offers advantages in the study of elastases that could be important in diseases such as arthritis,[6] pancreatitis,[1] atherosclerosis[3] and emphysema.[4] Although the lower limit of sensitivity of our assay is 150 picograms, it is possible to increase the sensitivity further by using less enzyme solution volume. For example, the use of 100 rather than 150 μl would increase the sensitivity to 100 picograms. In addition, it is theoretically possible to further enhance sensitivity by changing the geometry of the reaction vessel so that the volume/surface area ratio is decreased. This would increase interaction between soluble enzyme and insoluble substrate.

ACKNOWLEDGMENTS

Supported by NIOSH Grant OH-0092, American Lung Association and Medical Research Service, Cleveland VA Medical Center.

REFERENCES

1. M. Geokas, D. Murphy and R. McKenna. *Arch. Path.*, **86**, 117 (1968).
2. A. Janoff. in: *Neutral Proteases of Human Polymorphonuclear Leukocytes*, K. Havemann and A. Janoff (Eds.) Urban and Schwarzenberg, Baltimore-Munich, 1978, pp. 390–417.
3. A. Janoff. *Lab. Invest.*, **22**, 228 (1970).
4. G. Snider, J. Hayes, C. Franzblau, H. Kagan, P. Stone and A. Korthy. *Am. Rev. Respir. Dis.*, **110**, 254 (1974).
5. W. Bieger and G. Scheele. *Analyt. Biochem.*, **104**, 239 (1980).
6. D. Rifkin and R. Crowe. *Analyt. Biochem.*, **79**, 268 (1977).
7. S. Gordon, Z. Werb and Z. Cohn. in: *In Vitro Methods in Cell-Mediated and Tumor Immunity*, B. Bloom and J. Davis (Eds.), Academic Press, New York, 1976, pp. 348–350.
8. S. Takahashi, S. Seifter and F. C. Yang. *Biochim. Biophys., Acta.* **327**, 138 (1973).
9. P. Stone, G. Crombie and C. Franzblau. *Analyt. Biochem.*, **80**, 572 (1977).
10. M. Geokas, J. Brodrick, J. Johnson and C. Largman. *J. Biol. Chem.*, **252**, 61 (1977).
11. Y. Nakajima, S. Matsuno, N. Noto, Y. Saitoh and T. Sato. *Tohoku J. Exp. Med.*, **131**, 119 (1980).
12. H. Rinderknecht, M. Geokas, P. Silverman, Y. Lillad and B. Haverback. *Clin. Chim. Acta.*, **19**, 327 (1968).
13. L. Sachar, K. Winter, N. Sicher and S. Frankel. *Proc. Soc. Exp. Biol. Med.*, **90**, 323 (1955).
14. Z. Werb and S. Gordon. *J. Exp. Med.*, **142**, 361 (1975).
15. M. Schuyler and D. Steinberg. *J. Lab. Clin. Med.*, **100**, 932 (1982).
16. K. Walsh. in: *Methods in Enzymology*, Vol. 19, S. Colowick and N. Kaplan (Eds.), Academic Press, New York, 1970 pp. 41–63.
17. M. Banda and Z. Werb. *Biochem. J.*, **193**, 589 (1981).
18. M. Dahlgren, P. Davis and R. Bonney. *Biochim. Biophys. Acta.*, **630**, 338 (1980).
19. E. Levine, R. Senior and J. Butler. *Amer. Rev. Resp. Dis.*, **113**, 25 (1976).
20. Z. Werb. *J. Exp. Med.*, **147**, 1695 (1978).
21. A. White, H.-S. Lin and C. Kuhn. *J. Exp. Med.*, **146**, 802 (1977).
22. J. Bieth, N. B. Spiess and C. Wermuth. *Biochem. Med.*, **11**, 350 (1974).
23. L. Visser and E. Blout. *Fed. Proc.*, **28**, 207 (1969).
24. Z. Werb, M. Banda and P. Jones. *J. Exp. Med.*, **152**, 1340 (1980).