Methods of Enzymatic Analysis

Third Edition

Editor-in-Chief: Hans Ulrich Bergmeyer
Editors: Jürgen Bergmeyer and Marianne Graßl

Volume V
Enzymes 3: Peptidases, Proteinases and Their Inhibitors

Editorial Consultant: Hans Fritz

Weinheim · Deerfield Beach, Florida · Basel

Contents

2.9.3 Elastase-α_1-Proteinase Inhibitor Complex

Human granulocyte elastase (EC 3.4.21.37) complexed with α_1-proteinase inhibitor from plasma

Siegfried Neumann and Marianne Jochum

General

Human neutrophil leukocytes are rich in lysosomal neutral proteinases which are primarily responsible for intracellular protein breakdown in phagosomes. The best known enzyme of this group, an elastase, is one of the major constituents of the azurophilic granules of the polymorphonuclear leukocytes. As shown originally by *Janoff & Scherer* [1], granulocyte elastase can degrade elastin *in vitro* as well as various connective tissue proteins and plasma factors. Hence, the enzyme may play an important pathogenic role in tissue destruction and consumption of plasma factors, especially of clotting factors, if it is liberated extracellularly from the phagocytes during inflammatory diseases (for reviews, cf. [2]). Present evidence suggests that the amount of granulocyte elastase released extracellularly reflects the response of the organism to an inflammatory stimulus thus indicating the degree of participation of lysosomal factors in the inflammatory process.

In plasma samples, granulocyte elastase is found exclusively in complexes with plasma proteinase inhibitors [3, 4], primarily with the α_1-proteinase inhibitor (formerly called α_1-antitrypsin). Although approximately 10% of the elastase liberated may be bound to α_2-macroglobulin [3], the elastase-α_2-macroglobulin complex is normally hardly detectable in plasma samples due to the distinctly more rapid elimination of this complex ($t_{1/2} \sim 10$ min) from the circulation compared to the elastase-α_1-proteinase inhibitor complex ($t_{1/2} \sim 1$ h). Whereas the α_2-macroglobulin-bound elastase is still able to hydrolyze peptide substrates, the enzyme is totally inactive in the

complex with the α_1-proteinase inhibitor. However, elastase in this inactive complex is still accessible to specific antibodies.

Application of method: in clinical pathology, clinical chemistry, experimental cell research, haematology etc.

International reference methods and standards: not yet available.

Enzyme properties relevant in analysis: granulocyte elastase (EC 3.4.21.37) is a cationic glycoprotein with a molecular weight close to 30000 [4, 5]. Most of the enzyme preparations obtained so far can be separated into three multiple forms by ion-exchange chromatography [4, 5].

The three elastase forms give a reaction of identity in immunodiffusion and crossed immunoelectrophoresis [5]; they are also very similar with respect to their enzymatic or catalytic properties, at least towards synthetic peptide substrates. Elastase from human pancreas is a quite different protein which does not cross-react with antiserum against granulocyte elastase [6]. The same holds true for blood platelet elastase [7].

Granulocyte elastase reacts with its major natural antagonist in the human organism, the α_1-proteinase inhibitor, by forming a stable equimolar complex with a molecular mass of approximately 80000 [3, 4]. A minute transfer of the enzyme from this complex to free α_2-macroglobulin was reported by one group [3, 8]. The significance of this observation is not yet clear.

Methods of determination: elastase has been demonstrated immunologically in plasma samples of patients with septicaemia or leukaemia [9], in sera of myeloid leukaemia patients [10], in peritoneal fluid of peritonitis patients [11] and in sputum from bronchitis patients [12]. Immunochemical techniques described so far for elastase estimation are electro-immunodiffusion [9, 11], radioimmunoassay [6, 13], and a competitive type of solid-phase enzyme-linked immunoassay [14].

A sandwich type of solid-phase enzyme-linked immunoassay, which includes antisera against both elastase and α_1-proteinase inhibitor, was described recently [15a, b]*. Results on elastase/α_1-proteinase inhibitor levels in septicaemia or leukaemia [16] and rheumatoid arthritis [17] were reported previously. A version of this assay carried out in tubes is given below in detail.

Enzyme effectors: reversible inhibitors of active elastase include some long-chain fatty acids and polysaccharide sulphates, as well as the microbial peptide derivatives elastatinal and elasnin (for a review, cf. [18]). Irreversible inhibitors include the typical serine proteinase inhibitors diisopropyl fluorophosphate and phenylmethane sulphonylfluoride, and also methoxysuccinyl-Ala-Ala-Pro-Val-CH_2Cl which is a rather specific inhibitor of granulocyte elastase [18]. The soybean trypsin inhibitor (*Kunitz*) and inhibitory proteins from human seminal plasma and bronchial mucus, antileukoproteinase(s), are also known effectively to inhibit granulocyte elastase [19].

---

* Note added in proof: while this manuscript was in preparation an assay which is based on the same principle was published by a different group [24].

Assay

Method Design

Principle

(a) $spAb_E + EI \rightleftharpoons spAb_E\text{-}EI + EI$
$(B_1) \quad (F_1)$

(b) $spAb_E\text{-}EI + Ab_I^* \rightleftharpoons spAb_E\text{-}EI\text{-}Ab_I^* + Ab_I^*$
$(B_2) \quad (F_2)$

(c) 4-Nitrophenyl-P + $H_2O \xrightarrow{B_2}$ 4-nitrophenolate + P_i.

Sheep antibodies against elastase from human granulocytes are fixed to polystyrene tubes by adsorption. In reaction (a) the elastase inhibitor complex in either a standard solution or an unknown sample is reacted with the solid phase antibody. Free complex is then removed by washing the tubes. In reaction (b) the bound complex fraction from the first step (B_1) is reacted with the labelled antibody to α_1-proteinase inhibitor. Free labelled antibody is removed subsequently, and the bound label (B_2) is detected by incubation of the tubes with a chromogenic substrate in reaction (c). Substrate hydrolysis is stopped after a fixed time and absorption at 405 nm is measured. Assay blanks are made with sample diluent as a specimen.

Optimized conditions for measurement: calibrators and unknown samples are run in parallel and sample data are calculated from the calibration curve. Therefore, standardization with regard to temperature is optional within the range of 20 °C to 25 °C as long as all tubes within the series are treated in the same way. Since the immune reaction may not reach equilibrium within the incubation periods, the times of addition and removal of immunological reactants to and from the tubes have to be strictly controlled.

Equipment

Spectrophotometer or spectral-line photometer with precise measurement of absorbance at 405 nm, preferably with a flow cuvette for 0.5 or 1.0 ml; precision pipettes for 0.05, 1.0 and 2.0 ml; suction device (i.e. water pump etc.) with a trap; stopwatch.

$spAb_E$ solid phase fixed antibodies to granulocyte elastase.
EI complex of granulocyte elastase with α_1-proteinase inhibitor.
Ab_I^* antibodies to α_1-proteinase inhibitor with alkaline phosphatase as a label.
B_1, B_2 bound fractions from first or second reaction.
F_1, F_2 free fractions from first or second reaction.

Reagents and Solutions

Purity of reagents: all chemicals should be of the highest analytical grade commercially available. The antibodies against human granulocyte elastase should give only one precipitin line with extracts from leukocyte granules, i.e. they should react only with elastase and should not react with human plasma proteins. Suitable specific antisera against α_1-proteinase inhibitor can be obtained from different commercial suppliers. Purified immunoglobulin fractions of the rabbit antiserum are commercially available from *Dako*. The alkaline phosphatase used as a label is of the highest purity grade; suitable commercial preparations are available.

Preparation of reagents (for 96 determinations): all solutions in re-purified water (cf. Vol. II, chapter 2.1.3.2).

1. Antibody-coated tubes:

   prepare coating solution by dissolving 5 mg immunoglobulin fraction of sheep antiserum (e.g. from *Seward Lab.*, London, U.K.) against human granulocyte elastase in 100 ml NaCl, 0.15 mol/l (i.e. saline; dissolve 8.77 g NaCl in 100 ml water). Fill 96 polystyrene tubes (10.5 mm diameter and 40 mm height) with 1 ml coating solution and incubate at 2 °C to 8 °C for 24 h. Decant solution afterwards and rinse tubes three times with 2 ml saline. Aspirate the rest of the fluid carefully with the help of a water pump. Tubes are then ready for use. For prolonged storage the coated tubes may be washed finally with water, dried *in vacuo* and stored in airtight containers with a desiccant until use. Wash these tubes with saline once and aspirate all fluid before use.

2. Conjugate buffer (Tris, 0.01 mol/l, pH 7.5; NaCl, 0.15 mol/l; $MgCl_2$, 2 mmol/l; $ZnCl_2$, 0.025 mmol/l; dodecyl hydrogen sulphate, 1 mmol/l; bovine serum albumin, 10 g/l):

   dissolve 0.121 g Tris and 0.877 g NaCl in 50 ml water, adjust to pH 7.5 with HCl, 2 mol/l; then add 1 ml $MgCl_2$, 0.2 mol/l (4.066 g $MgCl_2 \cdot 6\ H_2O$ in 100 ml water) and 0.1 ml $ZnCl_2$, 25 mmol/l (0.341 g $ZnCl_2$ in 100 ml water); dissolve 29 mg dodecyl hydrogen sulphate, sodium salt, and 1 g bovine serum albumin and finally dilute to 100 ml with water.

3. Antibody-enzyme conjugate (1 mg/ml):

   antibodies against α_1-proteinase inhibitor are purified from rabbit antiserum by fractionation by ammonium sulphate precipitation and ion-exchange chromatography by an established procedure [20]. Alternatively, the immunoglobulin fraction of rabbit antiserum available from a commercial source (e.g. from *Dako* etc.) may be used. The antibodies are conjugated with calf intestinal alkaline phosphatase as described in the Appendix, p. 192. A final conjugate solution with an antibody titre of 1 mg antibody against α_1-proteinase inhibitor per litre and an alkaline phosphatase activity of approximately 700 U/l is used.

4. Sample diluent (phosphate, 0.01 mol/l, pH 7.5; NaCl 0.15 mol/l; EDTA, 20 mmol/l; bovine serum albumin, 1 g/l):

   dissolve 42.8 mg KH_2PO_4, 0.3 g $Na_2HPO_4 \cdot 2\ H_2O$, 1.75 g NaCl and 1.49 g EDTA in 50 ml water, adjust to pH 7.5 with NaOH, 1 mol/l, dissolve 0.2 g bovine serum albumin and dilute to 200 ml with water.

5. Standard solutions

   Stock solution

   Enzyme solution: dissolve 0.5 mg purified granulocyte elastase in 1 ml NaCl, 0.15 mol/l.

   Inhibitor solution: dissolve 2.8 mg α_1-proteinase inhibitor in 1 ml Tris/HCl, 60 mmol/l, pH 8.0, containing NaCl, 0.15 mol/l (0.725 g Tris and 0.877 g NaCl in 50 ml water, adjust to pH 7.5 with HCl, 2 mol/l, and dilute to 100 ml with water).

   Mix equal volumes of enzyme and inhibitor solution and incubate for 30 min at 37 °C. Check by polyacrylamide gel electrophoresis that the enzyme is totally complexed [4].

   Diluted standard solution: dilute stock solution of complex to a final concentration of 10 ng elastase/ml by serial dilution in sample diluent (4). Similarly, prepare standard solutions containing 5, 2.5 and 1 ng elastase in complex form per ml.

6. Washing solution (polyoxyethylene sorbitol monolaurate, 0.05% (w/v)):

   dissolve 0.25 g polyoxyethylene sorbitol monolaurate in 500 ml water.

7. AP assay reagents

7a. DEA buffer (1 mol/l, pH 9.8; Mg^{2+}, 0.5 mmol/l):

   dissolve 9.65 ml diethanolamine in 50 ml water, adjust to pH 9.8 with HCl, 1 mol/l, add 0.25 ml $MgCl_2$, 0.2 mol/l, and dilute to 100 ml with water.

7b. 4-Nitrophenyl phosphate (10 mmol/l):

   dissolve 371 mg 4-nitrophenyl phosphate, disodium salt, hexahydrate in 100 ml buffer (7a).

8. NaOH (2 mol/l):

   dissolve 8 g sodium hydroxide in 50 ml water, and dilute to 100 ml with water.

Stability of solutions: prepare 4-nitrophenyl phosphate solution (7b) freshly every day and store in the dark. Store standard solutions (5) at −20 °C until use and thaw once only. The antibody-coated tubes can be stored for 4 weeks at 2 °C to 8 °C without

decrease of quality when stored wet after rinsing as described under solution (1). Dried coated tubes can be stored for 1 year at 2 °C to 8 °C with negligible loss of function. All other reagents are stable for 4 weeks if kept in the refrigerator (+4 °C) and as long as no microbial contamination occurs.

Procedure

Collection and treatment of samples: collect blood from the vein into syringes or plastic tubes containing solid sodium citrate (3.8 mg sodium citrate for 1 ml) or EDTA (2 mg EDTA for 1 ml) as an anticoagulant. Do not shake blood vigorously during transport or storage. Store blood in cool conditions and remove plasma as quickly as possible.

Centrifuge for 10 min at about 3000 *g* and separate plasma from buffy coat so as not to disturb the buffy coat. Whereas heparinized plasma can also be used in this assay, serum cannot be used because elastase is liberated from granulocytes during clotting, thus causing falsely increased levels of the complex [22].

Stability of the analyte in the sample: the antigenic properties of the elastase/α_1-proteinase inhibitor complex in plasma are preserved for at least 24 h at 2 °C to 8 °C and for more than 6 months during storage at −20 °C. In samples from other sources (e.g. synovial fluids, effusions, seminal plasma etc.) the complex was found to be stable at −20 °C.

Assay conditions

Incubation

Dilute plasma or other specimen with sample diluent. It is most convenient to mix 50 µl plasma sample with 2.5 ml sample diluent in a polypropylene tube.

All measurements in duplicate.

A reagent blank with sample diluent (4) instead of sample is run.

Standard assays with standard solution (5) instead of sample are run, using 10, 5, 2.5 and 1 ng elastase in complex form per ml.

Start pipettings with blank, followed by standard and sample assays. It is essential to pipette in this sequence with constant time intervals throughout the incubation procedure.

It is not possible to indicate any concentration of reactants in the incubation mixture since reaction occurs on the solid phase.

Colour reaction

Wavelength 405 nm; light path 10 mm; final volume for absorbance reading 0.5 or 1.0 ml.

A blank is run with a mixture of 0.5 ml 4-NPP (solution (7b) and 0.5 ml NaOH, 2 mol/l.

A calibration curve is constructed with absorbances corrected for blank (ordinate) *versus* complexed elastase concentration (abscissa). A linear correlation should result.

Measurement

Pipette into coated tubes:	
sample diluent (4), diluted sample or standard solution (5)	0.5 ml
incubate 1 h at room temperature (20 °C to 25 °C), aspirate the contents	
washing solution (6)	2.0 ml
mix well, aspirate carefully, repeat washing two more times	
antibody-conjugate (3)	0.5 ml
incubate 1 h at room temperature, aspirate contents	
washing solution (6)	2.0 ml
aspirate contents carefully	
4-NPP solution (7b)	0.5 ml
incubate 90 min at room temperature in the dark	
NaOH solution (8)	0.5 ml
mix, transfer contents into cuvettes, read absorbance.	

Calculation: calculate mean values of duplicate measurements. A calibration curve is constructed on a linear graph paper with absorbances corrected for blank (ordinate) *versus* concentration of complexed elastase (abscissa), given as µg elastase per litre. A linear correlation should result. Calculate intercept at 0 µg/l and slope of the curve by linear regression.

Read concentrations of unknown samples from the calibration curve. The concentration in the undiluted analyte is calculated by multiplying the concentration in the diluted sample by the factor by which the analyte was diluted before the assay (e.g. factor 51 for plasma diluted by mixing 50 µl plasma with 2.5 ml sample diluent as described under "Assay conditions").

Validation of Method

Precision, accuracy, working range, detection limit and sensitivity: for two plasma pools with concentrations of about 50 µg/l and 200 µg/l granulocyte elastase complexed with α_1-proteinase inhibitor, the standard deviations were ± 4 and ± 8 µg/l. Within a series of 15 determinations the relative standard deviations (referred to the means values) were 7.7 and 3.9%, respectively. A coefficient of variation of approximately 8% was found for the between-series imprecision with 15 series. Data on accuracy are not available since standard reference material is not established yet. Standards mixed with plasma gave a recovery close to 100%.

The working range of the assay described is between 0.5 and 5 ng of elastase per assay tube, i.e. 1 to 10 ng/ml. When plasma diluted 1 : 51 is used this equals a range of 51 to 510 µg elastase per l undiluted plasma. The detection limit is defined as the mean absorbance of the reagent blank plus three times its standard deviation. It is about 0.25 ng elastase per test tube, i.e. 0.5 ng/ml. It can be lowered by increasing the incubation times for the immune reactions (a) and/or (b). The sensitivity corresponds to a $\Delta A = 0.005$ in the calibration curve and approaches 0.2 ng elastase/ml.

Sources of error: interference by improper handling of blood has been described [22, 23]. Addition of haemoglobin, lipids or rheumatic factors to plasma does not affect the assay [23]. Free α_1-proteinase inhibitor, which is present in high excess over the complex in plasma, does not interfere if sufficient washing is done following immune reaction (a) in the assay described above. Influences by therapeutics on the measured elastase concentrations are unknown so far [23].

Specificity: the concentration of the granulocyte elastase/α_1-proteinase inhibitor complex is measured specifically by making use of two distinct immunochemical reagents, specific antibodies directed towards granulocyte elastase and towards α_1-proteinase inhibitor. Elastase from pancreas or platelets and neutral proteinases other than granulocyte lysosomal elastase do not interfere in the assay.

Reference ranges: in human citrated plasma: 30 to 160 µg elastase/l [15b]. In another report a range between 10 and 160 µg elastase/l was found, with a median of 61 µg/l [23]. No difference was seen between sexes. Note that the given values represent the amount of elastase present in the complex and not the elastase-α_1-proteinase inhibitor complex in total.

Appendix

Preparation of antibody-enzyme conjugate

Reagents

Immunoglobulin

Purify the immunoglobulin fraction from rabbit antiserum against human α_1-proteinase inhibitor by ammonium sulphate fractionation and ion-exchange chromatography of antiserum according to a published procedure [20], or obtain as a commercial preparation (e.g. from *Dako* etc.).

Alkaline phosphatase

Use AP from calf intestine from commercial suppliers (e.g. *Boehringer Mannheim, Sigma* etc.) supplied as reagent grade, "for use as a marker in enzyme-linked immunoassays", in buffered solution.

Bovine serum albumin

Obtain the highest grade of purity commercially available.

Solutions

1. Phosphate-buffered saline (15 mmol/l, pH 7.5; NaCl, 0.135 mol/l):

   dissolve 320 mg KH_2PO_4, 2.25 g $Na_2HPO_4 \cdot 2H_2O$ and 7.89 g NaCl in 500 ml water, adjust to pH 7.5 with NaOH, 1 mol/l, and dilute to 1000 ml with water.

2. Glutardialdehyde reagent (2.1% (v/v); phosphate, 15 mmol/l, pH 7.5; NaCl, 0.135 mol/l):

   mix 0.1 ml 25% (v/v) glutardialdehyde (as obtained from commercial suppliers in sealed ampoules, highest grade of purity available) with 1.1 ml phosphate-buffered saline (1). Prepare this solution freshly before use.

3. Tris-buffered saline/Mg/Zn (Tris, 10 mmol/l, pH 7.5; NaCl, 0.15 mol/l; $MgCl_2$, 2 mmol/l; $ZnCl_2$, 0.025 mmol/l):

   dissolve 0.121 g Tris and 0.877 g NaCl in 50 ml water, adjust to pH 7.5 with HCl, 2 mol/l, then add 1 ml $MgCl_2$, 0.2 mol/l (dissolve 4.066 g $MgCl_2 \cdot 6H_2O$ in 100 ml water), add 0.1 ml $ZnCl_2$, 25 mmol/l (dissolve 0.341 g $ZnCl_2$ in 100 ml water), and dilute to 100 ml with water.

4. Conjugate buffer (Tris, 10 mmol/l, pH 7.5; NaCl, 0.15 mol/l; $MgCl_2$, 2 mmol/l; $ZnCl_2$, 0.025 mmol/l, dodecyl hydrogen sulphate, 1 mmol/l; bovine serum albumin, 10 g/l):

   dissolve 29 mg dodecyl hydrogen sulphate, sodium salt, and 1 g bovine serum albumin in 100 ml Tris-buffered saline/Mg/Zn solution (3).

Protein coupling

The antibody and alkaline phosphatase protein preparations are coupled by glutardialdehyde in an one-step procedure described previously [21].

Prepare the immunoglobulin in solution either by dissolving 10 mg (dry weight) purified immunoglobulin fraction in 1 ml phosphate-buffered saline (1), or by diluting immunoglobulin solution obtained commercially to a final concentration of 10 mg protein/ml using solution (1) as a diluent. Dialyze the solution against solution (1) at 2 °C to 8 °C for 48 h with one change of the dialysis buffer.

Dialyze the alkaline phosphatase solution against solution (1) as described for the antibody solution and finally adjust the enzyme to 1.2 mg protein/ml by dilution.

Mix 1 ml immunoglobulin solution with 4.2 ml dialyzed enzyme solution and add 0.26 ml glutardialdehyde reagent (2). Mix thoroughly and incubate without further shaking for 2 h at room temperature. Dialyze mixture against solution (1) overnight at 2 °C to 8 °C, then change dialysis buffer to Tris-saline/Mn/Zn solution (3) and dialyze for 48 h in the refrigerator.

Chromatography of the conjugate

Add the dialyzed coupling mixture to the top of a column (25 mm diameter × 1000 mm height) packed with an appropriate separation medium (e.g. Bio-Gel® A 1,5 m from *Bio-Rad Labs.*, Munich; Sephadex® G 200 (from *Pharmacia*) previously equilibrated with Tris/saline/Mg/Zn solution (3). Elute the sample with a flow rate of 4 ml × h^{-1} × cm^{-2} and collect fractions of 5 ml. Assay for activity of alkaline phosphatase in the eluate. The enzyme-labelled antibodies elute in the first enzyme peak which appears in the void volume. Peak fractions are pooled. Add dodecyl hydrogen sulphate, sodium salt, and bovine serum albumin to the pool to give final concentrations of 290 mg and 10 g, respectively, per 1 l of pool volume.

Characterization of the conjugate

Determine the antibody titre of the conjugate pool from the column and dilute with conjugate buffer (4) to a final concentration of approximately 1 mg of specific antibody against α_1-proteinase inhibitor per litre. Check by enzyme-immunoassay that this solution will give a suitable absorbance reading with the highest calibrator in the assay; in case of unacceptably high readings, dilute conjugate with a solution containing 1 mg of unlabelled antibody against α_1-proteinase inhibitor per litre.

Storage

Store conjugate in solution at 2°C to 8°C. To prevent microbial growth add appropriate preservatives, for instance sodium azide, 0.2 g/l, and organomercurials, 0.2 g/l.

Under these conditions the conjugate is stable for at least 12 months.

References

[1] *A. Janoff, J. Scherer,* Mediators of Inflammation in Leukocyte Lysosomes. IX. Elastinolytic Activity in Granules of Human Polymorphonuclear Leukocytes, J. Exp. Med. *128*, 1137–1151 (1968).

[2] *K. Havemann, A. Janoff* (eds.), Neutral Proteases of Human Polymorphonuclear Leukocytes, Urban & Schwarzenberg, Baltimore-Munich 1978.

[3] *K. Ohlsson, J. Olsson,* Neutral Proteases of Human Granulocytes. III. Interaction between Human Granulocyte Elastase and Plasma Protease Inhibitors, Scand. J. Clin. Lab. Invest. *34*, 349–355 (1974).

[4] *R. J. Baugh, J. Travis,* Human Leukocyte Granule Elastase: Rapid Isolation and Characterization, Biochemistry *15*, 836–841 (1976).

[5] *K. Ohlsson, J. Olsson,* The Neutral Proteases of Human Granulocytes. Isolation and Partial Characterization of Granulocyte Elastases, Eur. J. Biochem. *42*, 519–527 (1974).

[6] *K. Ohlsson, A.-S. Olsson,* Immunoreactive Granulocyte Elastase in Human Serum, Hoppe-Seyler's Z. physiol. Chem. *359*, 1531–1539 (1978).

[7] *Y. Legrand, J. P. Caen, L. Robert, J. L. Wantier,* Platelet Elastase and Leukocyte Elastase are Two Different Entities, Thromb. Haemostasis (Stuttg.) *37*, 580–582 (1977).

[8] *K. Ohlsson, C.-B. Laurell,* The Disappearance of Enzyme-inhibitor Complexes from the Circulation of Man, Clin. Sci. Mol. Med. *51*, 87–92 (1976).

[9] *R. Egbring, W. Schmidt, G. Fuchs, K. Havemann,* Demonstration of Granulocytic Proteases in Plasma of Patients with Acute Leukemia and Septicemia with Coagulation Defects, Blood *49*, 219–231 (1977).

[10] *L. Olsson, T. Olofsson, K. Ohlsson, A. Gustavsson,* Serum and Plasma Myeloperoxidase, Elastase and Lactoferrin Content in Acute Myeloid Leukaemia, Scand. J. Haematol. *22*, 397–406 (1979).

[11] *K. Ohlsson,* Collagenase and Elastase Released During Peretonitis are Complexed by Plasma Protease Inhibitors, Surgery *79*, 652–657 (1976).

[12] *R. A. Stockley, D. Burnett,* $Alpha_1$-Antitrypsin and Leukocyte Elastase in Infected and Non-infected Sputum, Am. Rev. Respir. Dis. *120*, 1081–1086 (1979).

[13] *E. F. Plow, J. Plescia,* Non-plasmin Mediated Fibrinolysis, in: *J. F. Davidson, J. M. Nilsson, B. Astedt* (eds.), Progress in Fibrinolysis V, Churchill Livingstone, Edinburgh, London, Melbourne, and New York 1981, pp. 70–74.

[14] *U. Kucich, W. B. Abrams, H. L. James,* Solid-phase Immunoassay of Dog Neutrophil Elastase, Anal. Biochem. *109*, 403–409 (1980).

[15a] *S. Neumann, N. Hennrich, G. Gunzer, H. Lang,* Enzyme-linked Immunoassay for Complexes of Human Elastase with α_1-Proteinase Inhibitor in Plasma, in: *D. M. Goldberg, M. Werner* (eds.), Progress in Clinical Enzymology *II*, Masson Publishers, New York 1983, pp. 293–298.

[15b] *S. Neumann, N. Hennrich, G. Gunzer, H. Lang,* Enzyme-linked Immunoassay for Elastase from Leukocytes in Human Plasma, J. Clin. Chem. Clin. Biochem. *19*, 232 (1981).

[16] *M. Jochum, K.-H. Duswald, E. Hiller, H. Fritz,* Plasma Levels of Human Granulocytic Elastase-α_1-Proteinase Inhibitor Complex (E-α_1PI) in Patients with Septicemia and Acute Leukemia, in: *D. M. Goldberg, M. Werner* (eds.), Selected Topics in Clinical Enzymology, de Gruyter, Berlin-New York 1983, pp. 85–100.

[17] *K. Kleesiek, S. Neumann, H. Greiling,* Determination of the Elastase α_1-Proteinase Inhibitor Complex, Elastase Activity and Proteinase Inhibitors in the Synovial Fluid, Fresenius Z. Anal. Chem. *311*, 434–435 (1982).

[18] *A. J. Barrett,* Leukocyte Elastase, in: *S. P. Colowick, N. O. Kaplan* (eds.), Methods in Enzymology, Vol. *80* part C, Academic Press, New York 1981, pp. 581 – 588.

[19] *H. Schiessler, K. Ohlsson, J. Olsson, M. Arnold, Y. Birk, H. Fritz,* Elastases from Human and Canine Granulocytes, II. Interaction with Protease Inhibitors of Animal, Plant, and Microbial Origin, Hoppe-Seyler's Z. physiol. Chem. *358*, 53 – 58 (1977).

[20] *N. Harboe, A. Ingild,* Immunization, Isolation of Immunoglobins, Estimation of Antibody Titre, Scand. J. Immunol. *2*, Suppl. 1, 161 – 164 (1973).

[21] *E. Engvall, K. Jonsson, P. Perlmann,* Enzyme-linked Immunosorbent Assay. II. Quantitation Assay of Protein Antigen, Immunoglobulin G, by Means of Enzyme-labelled Antigen and Antibody-coated Tubes, Biochim. Biophys. Acta *251*, 427 – 434 (1971).

[22] *E. F. Plow,* Leukocyte Elastase Release during Blood Coagulation. A Potential Mechanism for Activation of the Alternative Fibrinolytic Pathway, J. Clin. Invest. *69*, 564 – 572 (1982).

[23] *D. Neumeier, A. Fateh-Mogadham, G. Menzel,* Humane Granulocytenelastase. I. Zur Methodik einer enzymimmunologischen Bestimmung des Elastase-α_1-Proteinase-Inhibitor-Komplexes, Fresenius Z. Anal. Chem. *311*, 389 – 390 (1982).

[24] *M. Brower, P. Harpel,* Alpha-1-Antitrypsin-Human Leukocytes Elastase Complexes in Blood: Quantification by an Enzyme-Linked Differential Antibody Immunosorbent Assay and Comparison with Alpha-2-Plasmin Inhibitor-Plasmin Complexes, Blood *61*, 842 – 849 (1983).