PROTEINASE INHIBITORS

Medical and Biological Aspects

Edited by
Nobuhiko Katunuma, Hamao Umezawa, and Helmut Holzer

JAPAN SCIENTIFIC SOCIETIES PRESS Tokyo
SPRINGER-VERLAG Berlin Heidelberg New York Tokyo

1983

Preface

I have been studying antibiotics for the last 35 years, and whenever I report a new antibiotic, I receive many letters requesting a sample. Even more requests came to my office when I reported a new inhibitor of a protease. At present, I receive several letters each week requesting leupeptin, pepstatin, phosphoramidon, *etc.*, which indicated that many biochemists and biologists are involved in the study of proteases.

In the last 20 years, in the study of molecular biology great progress has been made, especially in the structure, function, and biosynthesis of DNA, RNA, and protein; and at the present, the study of proteases is increasing its importance. Moreover, proteases appear to be involved in all kinds of biological phenomena and disease processes. It is said that the number of researchers involved in protease studies is now increasing.

It seems that, in general, corresponding to each protease, there exists an inhibitor. It is certain that the study of proteases and their inhibitors is absolutely necessary for an understanding of biological functions and disease processes.

At present, the structures of these proteins are rapidly determined, and their structures can be displayed on a computer screen, being generated from data-base center. Even at this time, low mole-

cular weight inhibitors are contributing to the identification of proteases and their functions. Therefore, I think it was very fortunate that this book included not only the study of endogenous protease inhibitors but also of low molecular weight inhibitors. Also I am pleased that possible applications of these inhibitors for the treatment of diseases are discussed in this book. I should like to thank all contributors to this book, and I trust it will surely stimulate us to continue the search for new types of enzyme inhibitors.

July 1983

H. Umezawa

Hamao Umezawa

Editorial Note

During the past decade the importance of proteolytic processes in the regulation of intracellular protein metabolism and the post-translational processing of precursor proteins and peptide hormones has been recognized. Various pathological phenomena caused by disorders of these proteolytic processes have also been described. Because of this, increasing attention is being given to the rapid development of biochemical bases and applications of proteinase inhibitors.

Proteinase inhibitors can be classified into those which are synthetic and those occurring naturally, the latter obtained from microbial, plant, animal and other sources. From a biological aspect, these proteinase inhibitors can be separated into endogenous and exogenous. Synthetic and microbial proteinase inhibitors serve as exogenous inhibitors not only for the basic biochemistry and biology of proteolysis but also for various pathological phenomena and clinical application toward certain diseases. Proteinase inhibitors of animal origins play an important role in regulation of the self-defense mechanism, blood coagulation, intracellular protein catabolism and processing of precursor proteins. These inhibitors or their active fragments, if obtained, may find medical application in the future.

This monograph is a compilation of a substantial portion of the

recent knowledge gained in the medical and biological aspects of proteinase inhibitors. It is comprised of three sections. The biochemical and chemical bases of a number of microbial, plant and synthetic proteinase inhibitors are described in the first section. Their basic and clinical applications are also discussed. The second section includes topics on endogenous proteinase inhibitors from mammalian tissues and plasma. The role of these inhibitors in the regulation of proteinase activities and physiological functions is described. In the third section the biological and pathological significance of intracellular proteinases is detailed, and the role of these proteinases in intracellular protein degradation, defense mechanism and processing of precursor proteins is discussed. Thus the publication offers information on the most recent advances in this particular area of science in the fields of biochemistry, biology, and medicine.

On behalf of editors, I wish to cordially thank the authors for their cooperation and submission of manuscripts, and I also acknowledge Dr. P.C. Heinrich (Universität Freiburg) and Dr. E. Kominami (Tokushima University) for their assistance in the editing,

July 1983

Nobuhiko Katunuma

Contents

Microbial, Plant and Synthetic Inhibitors

A) Enzymological Aspects

Endogenous Proteinase Inhibitors

Regulation of Intracellular Proteinases

Proteinases and Their Inhibitors in Inflammation: Basic Concepts and Clinical Implication

M. Jochum,[*1] K.-H. Duswald,[*2] S. Neumann,[*3]
J. Witte,[*2] H. Fritz,[*1] and U. Seemüller[*1]

*Department of Clinical Chemistry and Biochemistry, University of Munich,[*1]
Surgical Clinic, University of Munich,[*2] and Department of Biochemical Research,
E. Merck[*3]*

THE ROLE OF LYSOSOMAL ENZYMES IN INFLAMMATION

During the inflammatory response various systemic or local tissue cells are activated thereby releasing internal, mostly lysosomal enzymes. They trigger the activation of the clotting, fibrinolysis and complement cascades, the disruption of cell membranes and tissue structure, and the release of toxic peptides (Fig. 1).

Phagocytes, especially the granulocytes and monocytes or macrophages, but also fibroblasts, endothelial cells, and mast cells are known to be very rich in such internal or lysosomal enzymes. So far, only the properties and pathobiochemical effects of enzymes of the azurophilic and specific lysosomes of polymorphonuclear granulocytes (neutrophils) have been investigated in more detail. Such enzymes, for example the neutrophil elastase and cathepsin G as well as the acidic cathepsins are preformed and stored in the lysosomes in fully active form (7, 11). In this way, they can respond immediately to perform their biological

[*1,*2] D-8000 München 2, FRG.
[*3] D-6100 Darmstadt 1, FRG.
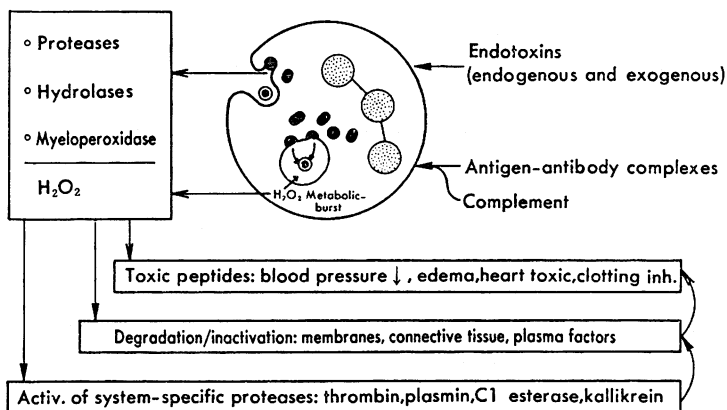
Fig. 1.   Liberation and effects of lysosomal factors. For details see text.
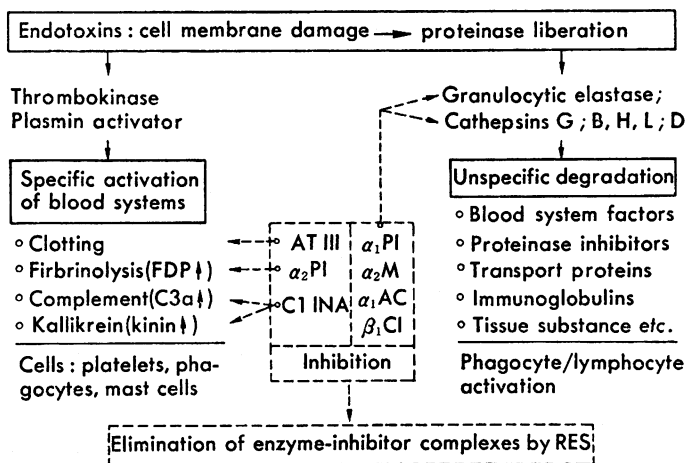
Fig. 2.   Consumption of plasma factors during inflammation.
AT III, antithrombin III; α_2PI, α_2-plasmin inhibitor; C1 INA, C1 inactivator; α_1PI, α_1-proteinase inhibitor (formerly α_1-antitrypsin); α_2M, α_2-macroglobulin; α_1AC, α_1-antichymotrypsin; β_1CI, β_1-collagenase inhibitor; FDP, fibrin(-ogen) degradation products; C3a, complement factor. For details see text.

function, namely, the degradation of extracellular and intracellular material after phagocytosis.

Outside the phagocytes, proteolytic action of the lysosomal enzymes is normally prevented or balanced by proteinase inhibitors present in plasma, interstitial fluid and body secretions. Non-lysosomal

proteinases like, for example, thrombokinases and plasminogen activators are faced only with a very low inhibitory potential in body fluids. They are, therefore, privileged candidates for the activation of clotting and fibrinolysis if released into the circulation after increased production due to an inflammatory stimulus.

Lysosomal enzymes liberated during severe inflammation like septicemia or septic shock can enhance, together with thrombokinases and plasminogen activators, the inflammatory response *via* two major routes characterized by either substrate-specific or substrate-unspecific proteolysis (Fig. 2).

The system-specific proteinases, thrombokinases and plasminogen activators, trigger the activation of the clotting, fibrinolysis and complement cascades (summarized as 'blood systems') by *substrate-specific* proteolysis of proenzymes and cofactors. The activated enzymes are subsequently inhibited by their natural inhibitors; the enzyme-inhibitor complexes thus formed are rapidly eliminated from the circulation by the reticuloendothelial system (RES). Hence, in a series of steps based on highly specific interactions not only the proteinases respectively their zymogens are consumed but also their natural antagonists, the system-specific inhibitors. Until recently, the given sequence of reactions was assumed to be exclusively responsible for the development of disseminated intravascular coagulation (DIC).

Results obtained very recently indicate that an additional reaction path may contribute considerably to the consumption of plasma factors during severe inflammations. This implies inactivation of plasma factors by *substrate-unspecific* proteolysis due to liberated lysosomal proteinases. Egbring and coworkers observed in animals a significant decrease in several clotting factors after infusion of endotoxin or human neutrophil elastase (4). Similar results were obtained by Ohlsson and coworkers (1, 2) in canine endotoxemia. Moreover, in patients suffering from sepsis or septic shock a striking consumption of blood system factors, immunoglobulins and proteinase inhibitors has been found by the teams of Egbring (5), Aasen (3), Gallimore (6), and Witte (14).

NEUTROPHIL ELASTASE AND PLASMA FACTORS IN SEPSIS

In the following study neutrophil elastase was chosen as a marker

enzyme in order to demonstrate the release of lysosomal enzymes during the development of septicemia.

1. Assay of Liberated Elastase

Due to the presence of an excess of the endogenous inhibitors α_1-proteinase inhibitor (α_1PI) and α_2-macroglobulin (α_2M), direct measurement of the neutrophil-derived proteinase activities in plasma or other body fluids is not feasible. However, increased levels of the elastase-α_1PI (E-α_1PI) complex would be already a clear indication for elastase liberation. Quantitative estimation of the plasma levels of the E-α_1PI complex was carried out with a highly sensitive enzyme-linked immunoassay (12). Briefly, the E-α_1PI complex of the plasma sample was bound to surface-fixed antibodies directed against neutrophil elastase. After washing, a second alkaline phosphatase-labelled antibody directed against α_1PI was fixed to the complex. Under suitable conditions, the activity of fixed alkaline phosphatase towards p-nitrophenylphosphate is proportional to the concentration of the E-α_1PI complex in the sample.

In a first approach, we were interested to see whether a relationship exists between the plasma levels of E-α_1PI and the severity of postoperative infections. To achieve this purpose, besides other factors the levels of factor XIII (Faktor XIII-Schnelltest, Behringwerke AG Marburg) and antithrombin III (AT III) (S-2238, Deutsche Kabi Munich) were continuously monitored because both clotting factors are known to be easily degraded by neutrophil elastase *in vitro* (9).

2. Patients

In the clinical trial, patients subjected to major abdominal surgery were included if the operation time exceeded 120 min. Diagnosis of septicemia in the postoperative course was confirmed by prospectively established septic criteria:

  Defined infection site and pos. bact. culture
  Body temperature $> 38.5°C$
  Leukocytosis with $> 15,000$ cells/mm^3 or
  Leukocytopenia with $< 5,000$ cells/mm^3
  Platelets $< 100,000$/mm^3 or drop $> 30\%$
  (Positive blood culture)

In the prospective study more than 120 patients were included. Thirty of them fulfilled the defined septic criteria during the postoperative course. Of these patients, fourteen survived the infection (group B) whereas sixteen died as a direct result of septicemia (group C). Eleven patients being without infection after abdominal surgery served as controls (group A).

3.   E-α_1PI Levels

With the enzyme-linked immunoassay elastase levels between 60 and 110 ng/ml were found in 153 healthy individuals. In patients without preoperative infection (groups A and B), the operative trauma was followed by an increase of the E-α_1PI level up to 3-fold of the normal value. Patients suffering from preoperative infections (6 out of 16 in group C) showed already clearly elevated preoperative E-α_1PI levels. Immediately after surgery a slight decrease was observed, probably due to elimination of the infection focus. Before onset of sepsis, the E-α_1PI concentrations of group B and C showed a moderate elevation but no significant changes compared to the postoperative levels. However, at the beginning of septicemia a highly significant increase of the E-α_1PI
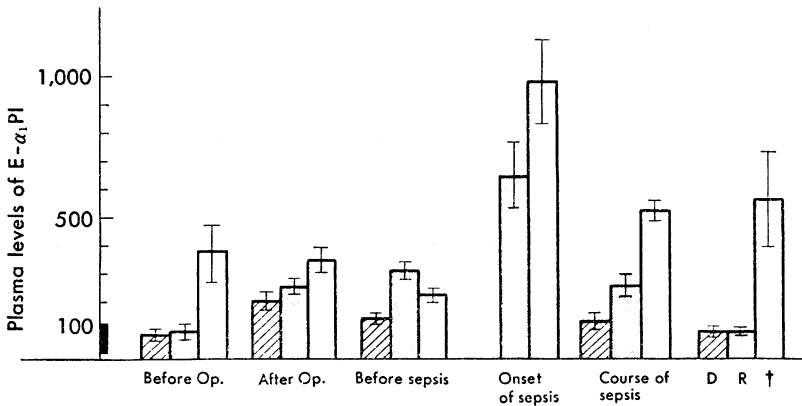
Fig. 3.   Plasma levels of E-α_1PI in patients subjected to major abdominal surgery. ▨ (A) patients ($n=11$) without postoperative infection. ▦ (B) patients ($n=14$) surviving postoperative septicemia. ☐ (C) patients ($n=16$) dying as a result of septicemia. The E-α_1PI levels are given as mean values ($\pm$S.E.M.) for the day before operation, the day after operation as well as for the postoperative phase before sepsis, at onset of sepsis and during septicemia. Last determinations were done on day of discharge (D) for group A, on day of recovery (R) for group B, and before death (†) for group C.

levels could be detected: up to 6-fold in group B and up to 10-fold in group C. Peak levels were found above 2,500 ng/ml in both groups. The E-α_1PI levels of septic patients who recovered showed a clear tendency towards normal values. In patients with persisting septicemia, high levels of E-α_1PI were measured until death (Fig. 3).

4. AT III Activity

In non-infected patients the activity of AT III, the most important inhibitor of the clotting system, was in the normal range during the whole observation period. In infected patients, however, the AT III activity was found already below the clinically critical concentration of about 75% of the standard mean value before onset of septicemia. This low value normalized in all patients overcoming the infection, whereas a further significant decrease (up to 45% of the norm) was found in group C patients with lethal outcome. Probably, the extremely low AT III activity in the latter patients, having permanently elevated E-α_1PI levels, may be due to a significant degree to degradation by lysosomal enzymes and especially by elastase.

5. Factor XIII Activity and A and S Subunit Levels

Similar results were obtained for factor XIII, the fibrin stabilizing co-agulation factor. In plasma of patients who did not survive septicemia, the factor XIII activity decreased up to 28% of the standard mean value. As measured by immunoelectrophoresis, these patients also had very low concentrations of both subunit A, comprising the active enzyme, and subunit S, representing the carrier protein (data not shown). In contrast, group A patients with an uncomplicated postoperative course showed normal or only slightly decreased concentrations of subunit S, although subunit A and fibrin stabilizing activities were often significantly reduced.

As demonstrated earlier by Egbring and coworkers (5) and Ikematsu and coworkers (8), reduction of both subunits of factor XIII cannot be due to activation of the clotting cascade alone. During clotting, that means by the action of thrombin only subunit A is consumed simultaneously with the factor XIII activity but not subunit S. Elastase, however, is able to degrade both subunits to a similar degree. These data and the results presented in our clinical trial suggest that in the
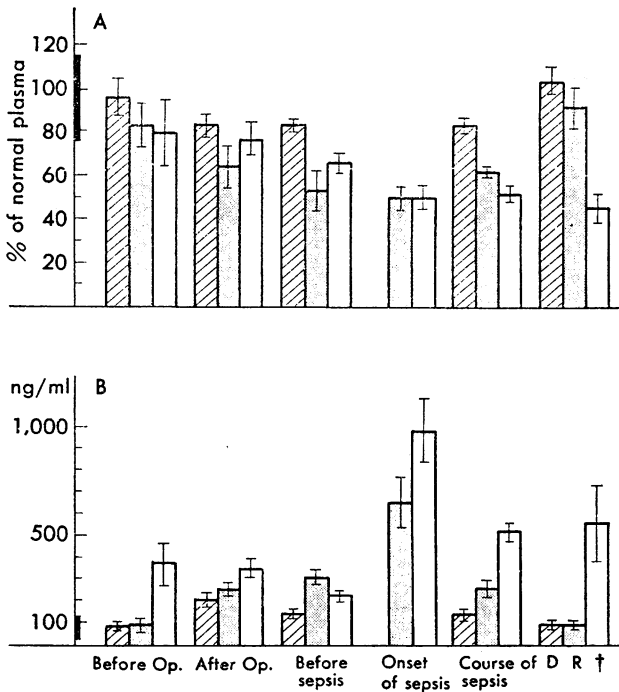
Fig. 4. Plasma levels of the inhibitory activity of AT III (A) compared to the amount of the E-α_1PI (B) in patients subjected to major abdominal surgery. For details, see legend to Fig. 3.

patients suffering from septicemia, unspecific proteolytic degradation by granulocytic elastase and/or other lysosomal proteinases is involved to a significant degree in the depletion of factor XIII.

6. Conclusion from the Clinical Studies

The results of the clinical studies show that in inflammatory diseases a correlation exists between the release of a lysosomal enzyme marker, the neutrophil elastase, and the clinical situation of the patient, respectively, the consumption of selected plasma factors. We take this as a clear indication that liberated lysosomal factors and especially neutrophil proteinases contribute significantly to the inflammatory response of the organism by substrate-unspecific degradation of plasma and other factors. Early application of suitable and potent exogenous proteinase
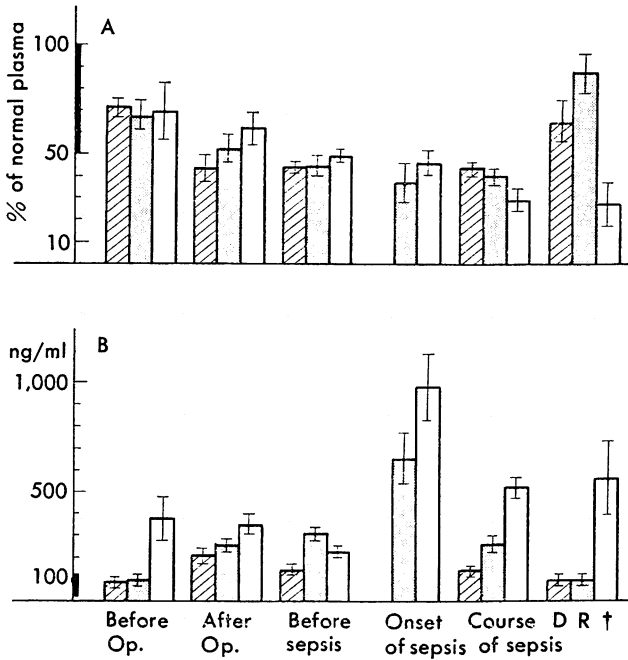
Fig. 5.   Plasma levels of the fibrin stabilizing activity of factor XIII (F XIII) (A) compared to the amount of the E-a_1PI (B) in patients subjected to major abdominal surgery. For details, see legend to Fig. 3.

inhibitors should prevent or at least diminish, therefore, such destructive proteolytic processes.

INHIBITOR THERAPY IN EXPERIMENTAL ENDOTOXEMIA

To confirm this assumption, we established an endotoxemia model in dogs by intravenous infusion of *Escherichia coli* endotoxin for 2 hr. Thereby, a significant decrease was observed in the plasma levels of the clotting factors AT III, prothrombin, and factor XIII, of the fibrinolysis factors plasminogen and α_2-antiplasmin, and of the complement factor C3. The levels were followed up over an experimental period of 14 hr and their alterations checked for statistical significance (*10*) (Fig. 6). Simultaneous intravenous administration of a relatively specific inhibitor of neutrophil elastase and cathepsin G, the Bowman-Birk

Fig. 6. Changes in the plasma levels of various plasma factors during the acute phase of experimental endotoxemia in dogs ($n=6$ for each group) without or with inhibitor treatment.
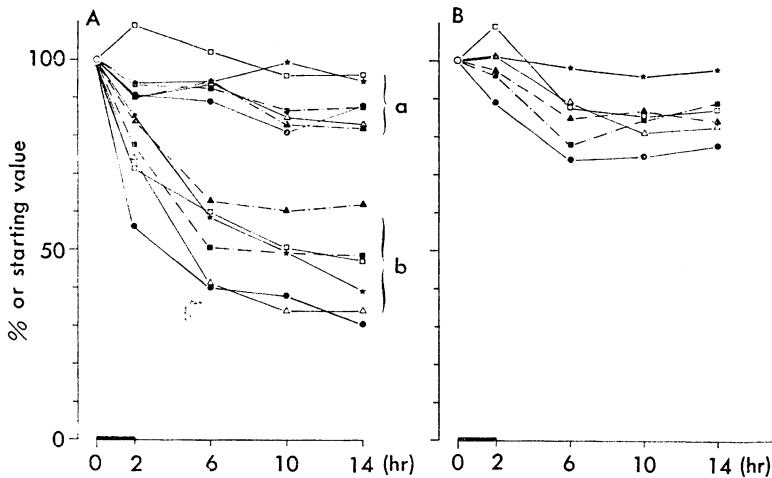Applied inhibitor dosages: 10–25 trypsin inhibiting units (Bowman-Birk inhibitor) per kg body weight over the observation period of 14 hr. Data are given as mean values in percentage of the individual starting values. Thick line at the abscissa = endotoxin infusion period. For further details see text and ref. *10*. A: a, controls, b, endotoxin-treated. B: inhibitor and endotoxin-treated. ● complement C3; ★ factor XIII; △ prothrombin; ▲ AT III; □ plasminogen; ■ antiplasmin.

inhibitor from soybeans, clearly reduced the endotoxin-induced decline of the tested plasma factors. The inhibitor (*Mr* 7,000) was effective in dosages ranging from 3–8 mg (*i.e.*, 10–25 trypsin inhibiting units) per kg body weight. A reasonable assumption would be, therefore, that the exogenous inhibitor was able to prevent or reduce the neutrophil proteinase-induced consumption reactions very effectively.

EGLIN, A POTENT INHIBITOR OF NEUTROPHIL PROTEINASES

Another promising candidate for an inhibitor therapy in inflammatory processes might be eglin, an inhibitor present in the leech *Hirudo medicinalis*. It is a mini inhibitor protein with a molecular weight of 8,100 consisting of a single peptide chain with 70 amino acid residues (Fig. 7) (*13*). Eglin inhibits very strongly the neutral granulocytic proteinases elastase and cathepsin G, as well as chymotrypsin and sub-

1   10   20
NH₂-Thr -Glu-Phe-Gly-Ser -Glu-Leu -Lys -Ser-Phe-Pro -Glu-Val -Val-Gly-Lys -Thr -Val-Asn-Gln-

                                    30   40
Ala-Arg-Glu-Tyr- Phe-Thr-Leu-His -Tyr-Pro-Gln-Tyr-Asp-Val-Tyr- Phe-Leu -Pro-Glu-Gly-

                                    50   60
Ser -Pro -Val-Thr- Leu -Asn-Leu -Arg-Tyr-Asn-Arg-Val-Arg-Val-Phe-Tyr -Asn-Pro- Gly-Thr-

                        70
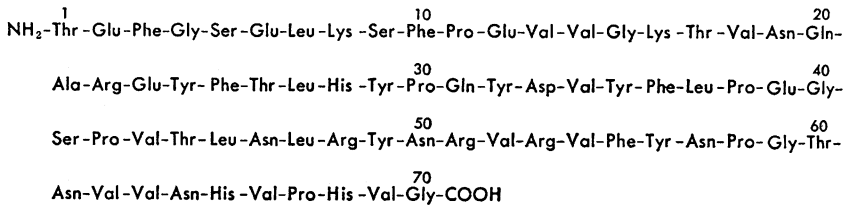Asn-Val -Val-Asn-His -Val-Pro-His -Val-Gly-COOH

Fig. 7. Primary structure of eglin, an inhibitor of neutrophil elastase and cathepsin G from the leech *H. medicinalis*.

tilisin. The dissociation constants are in the range of 10^{-10} M. Eglin might be a useful drug in inflammatory processes which are at least partially caused by neutral granulocytic proteinases.

GENERAL CONCLUSIONS

In severe inflammatory processes, multiple trauma or shock, various cells such as neutrophils, macrophages, endothelial cells, and mast cells are stimulated or disintegrated. In this way a high potential of lysosomal enzymes is released of which the proteinases are of special pathogenetic effectiveness. Recent studies in our laboratory and by others indicate strongly that *substrate-unspecific proteolysis* by lysosomal proteinases and especially by the neutrophil elastase contributes to a significant degree to the consumption and/or degradation of extracellular substances in such diseases. On the other hand, early administration of convenient exogenous inhibitors directed against the lysosomal enzymes should have a positive therapeutic effect also in humans.

REFERENCES

1. Aasen, A. O., Ohlsson, K., Larsbraaten, M., and Amundsen, E. *Eur. J. Surg. Res.*, **10**, 63 (1978).
2. Aasen, A. O. and Ohlsson, K. *Hoppe-Seyler's Z. Physiol. Chem.*, **359**, 683 (1978).
3. Aasen, A. O., Smith-Erichsen, N., Gallimore, M. J., and Amundsen, E. *Adv. Shock Res.*, **4**, 1 (1980).

4. Egbring, R., Gramse, M., Heimburger, N., and Havemann, K. *Diath. Haemorrh.*, **38**, 222 (1977).
5. Egbring, R., Schmidt, W., Fuchs, G., and Havemann, K. *Blood*, **49**, 219 (1977).
6. Gallimore, M. J., Aasen, A. O., Lyngaas, K., Larsbraaten, M., Smith-Erichsen, N., and Amundsen, E. *Microvasc. Res.*, **18**, 292 (1979).
7. Havemann, K. and Janoff, A. *In* "Neutral Proteases of Human Polymorphonuclear Leukocytes," eds. K. Havemann and A. Janoff (1978). Urban und Schwarzenberg Verlag, Baltimore-Munich.
8. Ikematsu, S., McDonagh, R. P., Reisner, H. M., Skrzynia, C., and McDonagh, J. *J. Lab. Clin. Med.*, **97**, 662 (1981).
9. Jochum, M., Lander, S., Heimburger, N., and Fritz, H. *Hoppe-Seyler's Z. Physiol. Chem.*, **362**, 103 (1981).
10. Jochum, M., Witte, J., Schiessler, H., Selbmann, H. K., Ruckdeschl, G., and Fritz, H. *Eur. Surg. Res.*, **13**, 152 (1981).
11. Klebanoff, S. J. and Clark, R. A. *In* "The Neutrophil Function and Clinical Disorders," eds. Klebanoff and Clark (1978). Elsevier/North-Holland Biomedical Press, Amsterdam.
12. Neumann, S., Hennrich, N., Gunzer, G., and Lang, H. *In* "Progress in Clinical Enzymology-II," eds. D. M. Goldberg and M. Werner (1983). Masson Publishing USA, Inc., in press.
13. Seemüller, U., Fritz, H., and Eulitz, M. *Methods Enzymol.*, **80**, 804 (1981).
14. Witte, J., Jochum, M., Scherer, R., Schramm, W., Hochstrasser, K., and Fritz, H. *Intensive Care Med.*, **8**, 215 (1982).