Hoppe-Seyler's Zeitschrift für Physiologische Chemie

45. Konferenz der Gesellschaft für Biologische Chemie

W DE G

Herausgegeben von

K. Decker, W. Stoffel, H.G. Zachau

[illegible]r Mitwirkung von H. Eggerer, J. Engel, H. Fritz, E. Habermann, E. Helmreich, A. Henschen, B. H[illegible]

[illegible]ilschmann, H. Hilz, P. W[illegible] [illegible]blut, K. Jungermann, P. Karlson, H. Kleinkauf, H.L. Kornberg, K. K[illegible]

Oeste[illegible] [illegible]ndhoff, R. Schauer, J. Seelig, G. Siebert, H. Sies, H. Simon, E. Trusche

H. [illegible] Ullrich, E. Wecker, H. Wiegandt, B. Wittmann-Liebold, H. Zahn

[illegible]edaktion A. Dillmann, G. Peters

E GRUYTER · BERLIN · NEW YORK

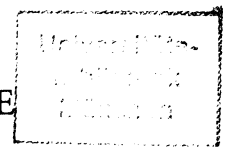
Hoppe-Seyler's Z. Physiol. Chem. Bd. 364, Heft 7 S. 777–922 Juli 1983
HSZPAZ 364 (7) 777–922 (1983)

BIOCHIMIE

Publication mensuelle
éditée par la SOCIÉTÉ de CHIMIE BIOLOGIQUE
4, Avenue de l'Observatoire, 75270 Paris Cedex 06

Abonnements: 920 FF - 200 US $

1983 - TOME 65 - N° 4-5



SOMMAIRE

Hoppe-Seyler's Z. Physiol. Chem.
Bd. 364, S. 1709–1715, Dezember 1983

Inter-α-Trypsin Inhibitor of Human Serum:

An Inhibitor of Polymorphonuclear Granulocyte Elastase

Marianne JOCHUM and Astrid BITTNER

Abteilung für Klinische Chemie und Klinische Biochemie in der Chirurgischen Klinik Innenstadt der Universität München

(Received 25 July/24 October 1983)

Summary: Inter-α-trypsin inhibitor (ITI) was separated from human serum in a single step using ion-exchange chromatography via the fast protein liquid chromatographic system. The inhibitor preparation, identified as ITI by specific immunological assays, inhibited human polymorphonuclear granulocytic elastase besides bovine trypsin. Hence, ITI is the third candidate of the plasma proteinase inhibitors (in addition to α_1-proteinase inhibitor and α_2-macroglobulin) capable of inhibiting lysosomal granulocytic elastase.

Inter-α-trypsininhibitor aus Humanserum: Ein Hemmstoff der Elastase aus polymorphkernigen Granulozyten

Zusammenfassung: Inter-α-trypsininhibitor (ITI) wurde durch Ionenaustauschchromatographie mittels des „Fast Protein Liquid Chromatography"-Systems in einem Schritt aus Humanserum separiert. Die anhand immunologischer Methoden als ITI identifizierte Inhibitorfraktion hemmte außer Trypsin auch Elastase aus menschlichen polymorphkernigen Granulozyten. Demnach ist ITI, neben α_1-Proteinaseinhibitor und α_2-Makroglobulin, der dritte Kanditat der Plasmaproteinaseninhibitoren für die Hemmung lysosomaler granulozytärer Elastase.

Key words: PMN granulocytic elastase, inter-α-trypsin inhibitor, fast protein liquid chromatography system.

Enzymes:
Polymorphonuclear granulocyte elastase (EC 3.4.21.37); trypsin (EC 3.4.21.4).

Abbreviations:
α_1-PI = α_1-proteinase inhibitor (formerly α_1-antitrypsin);
Bistris = 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol;
BzArgNan = benzoyl-L-arginine-4-nitroanilide;
FPLC = fast protein liquid chromatography;
Hepes = 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid;
ITI = inter-α-trypsin inhibitor;
IU = inhibitor units;
PMN = polymorphonuclear;
Suc[Ala]$_2$ValNan = 3-carboxypropionyl-L-alanyl-L-alanyl-L-valine-4-nitroanilide;
TosPheCH$_2$Cl = (*N*-*p*-toluenesulfonyl-L-phenylalanyl)chloromethane;
Tricine = *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

Inter-α-trypsin inhibitor (ITI), although only a minor proteinase inhibitor in human plasma or serum[1], has a broad inhibition spectrum[1,2]. It reacts mainly with trypsin, curiously showing a higher affinity for bovine than for the human enzyme. The same holds true for bovine and human chymotrypsin[3]. In contrast, acrosin, a trypsin-like enzyme from spermatozoa, reacts with ITI with a higher affinity for the human than for the animal (boar) enzyme[4]. Whereas a weak inhibition of human plasmin by ITI was also demonstrated[1,2], inhibition of PMN-granulocytic elastase by ITI still remains a matter of controversial discussion[2,5,6]. Only recently, Jönsson et al.[5] and Bromke and Küppers[6] demonstrated inhibition of granulocytic elastase by urinary trypsin inhibitors of lower molecular mass cross-reacting immunologically with ITI from human serum. On the other hand, Hochstrasser's group[2,7] has not up to now demonstrated such an elastase specificity either for isolated serum ITI or its cleavage products isolated from urine or obtained artificially by trypsinolysis. As ITI is known to be a very labile protein[1,6,7] it is likely that the native, fully active inhibitor molecule can be isolated only with gentle techniques especially avoiding strong acidic conditions. Despite several attempts[6] in this respect, a successful rapid separation of ITI from human serum has not been achieved so far. Taking advantage of a newly developed isolation system [fast protein liquid chromatography (FPLC System)], we have now been able to separate ITI from serum proteins in a single, rapid step and to demonstrate its inhibitory activity towards PMN-granulocytic elastase.

Material and Methods

a) Ion exchange chromatography

Separation of ITI from 1 or 5 ml human serum was achieved within 20–30 min using the FPLC System (Pharmacia, S-75104 Uppsala) with a pre-packed anion-exchange column, Mono Q HR 5/5. The NaCl gradient was established with Bistris buffer A (20mM, pH 6.2) and B (A + 1.0M NaCl). The procedure followed was that suggested by the manufacturer and will be published in detail elsewhere (Jochum, M., Bittner, A. & Gunzer, G., in preparation). For comparison, pre-purified ITI (~ 80% purity; research product from Behringwerke, D-3550 Marburg) and α_1-proteinase inhibitor [α_1-PI, immunologically homogenous against antihuman plasma antisera protein (Dako, C.H. Boehringer, D-6507 Ingelheim), research product from E. Merck, D-6100 Darmstadt] were subjected to the chromatographic procedure under identical conditions.

b) Enzyme activity and inhibition assays

Granulocytic elastase: Human PMN-granulocytic elastase was isolated according to the method of Baugh and Travis[8]. Elastase amidolytic activity and its inhibition were measured by minor modifications of the method published by Wenzel et al.[9] using the chromogenic substrate Suc[Ala]$_2$ValNan (Bachem, Bubendorf, Switzerland). Briefly, 30 µl enzyme solution (0.5 µg elastase dissolved in 0.1M CH_3CO_2Na, 0.05% Triton X-100, pH 6.5) was incubated at 25 °C with 870 µl (– x µl inhibitor solution) 0.1M Hepes buffer pH 8.3, containing 2M NaCl and 0.05% Triton X-100. The enzyme activity was determined after addition of 100 µl substrate solution (10mM in 100% dimethylsulfoxide) at 405 nm.

Bovine trypsin: Bovine trypsin (treated with TosPhe-CH_2Cl, Merck) activity and its inhibition were determined with BzArgNan (Merck) as substrate, according to Fritz et al.[10].

c) Immunoelectrophoresis

Rocket immunoelectrophoresis was performed according to Laurell[11] in 1% agarose on plastic plates from LKB (S-161 25 Bromma) using agarose tablets and Tris/Tricine buffer pH 8.6 (0.08M Tris, 0.024M Tricine, 0.3mM calcium lactate, 0.02% NaN_3; from Bio-Rad Laboratories, Richmond, CA). Electrophoresis was carried out at 8–10 V/cm for 4 h. The specific antisera against ITI or α_1-PI were purchased from Behringwerke and applied in a final concentration of 1% and 1.5%, respectively. 5 µl of antigen samples were inserted into the gels and the plates stained with 0.5% Coomassie Brillant Blue R 250 (Serva, D-6900 Heidelberg) for 45 min.

d) Dodecyl sulfate polyacrylamide gel electrophoresis

Dodecyl sulfate gel electrophoresis under reducing (with 1% 2-mercaptoethanol) or non-reducing conditions was performed as horizontal flat gel electrophoresis with the LKB 2117 Multiphor apparatus according to the LKB application note 306. The thickness of the 5% and 7.5% gels were 2 mm each, the applied sample volumes 10 µl. For molecular mass calibration high (40–250 kDa) and low (10–100 kDa) standard protein mixtures from Bio-Rad Laboratories were used. The gels were subjected to electrophoresis at 8–9 V/cm for 3 h. After protein fixation for 30 min with 20% (w/v) trichloroacetic acid staining was performed with 0.15% Coomassie Brillant Blue R 250 for 2 h.

Results and Discussion

Ion-exchange chromatography is commonly used during purification of ITI from various body fluids (for a review, see ref.[12]). The essential advantage of the procedure described here (FPLC System) is the extremely short separation time, thereby probably avoiding degradation and partial inactivation of the native inhibitor molecule[1,6,7,12].

The primary aim of this work was to show inhibitory activity of ITI towards granulocytic elastase, after complete separation from α_1-PI present in serum in a 20-fold molar excess over ITI[1]. To determine the exact elution volumes

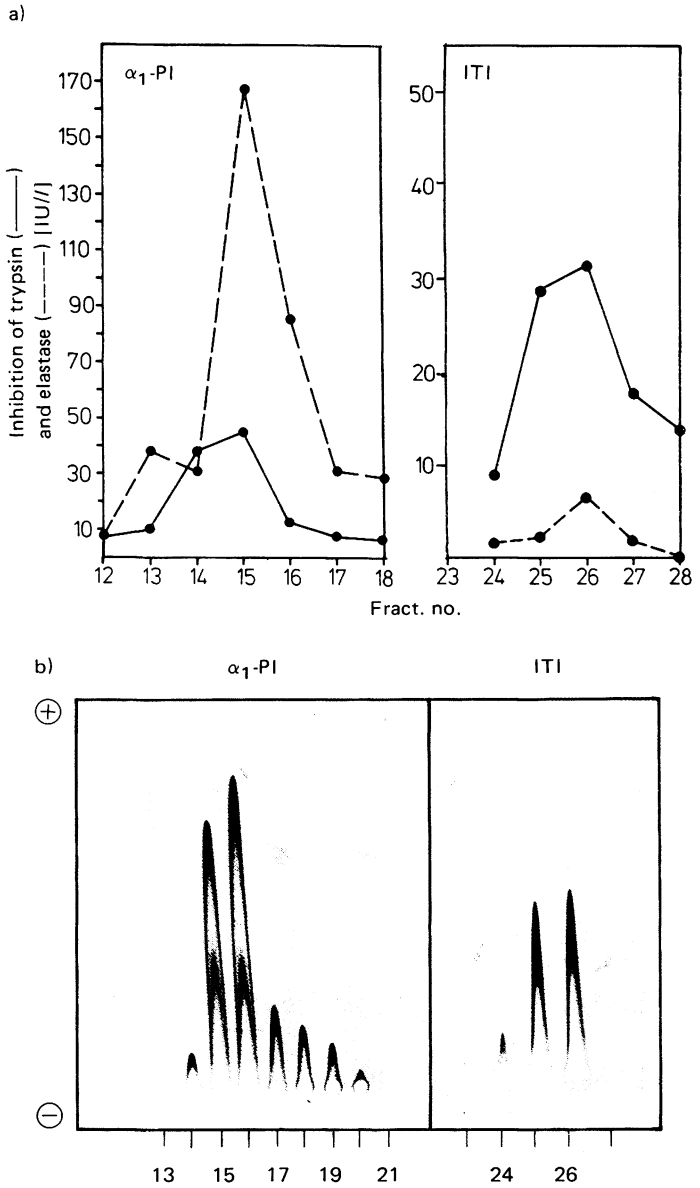


Fig. 1. Separation of the prepurified inhibitors ITI and α_1-PI with the FPLC System.
a) The elution diagrams representing inhibition of trypsin (•——•) and granulocytic elastase (•– – –•) by α_1-PI and ITI were chromatographed separately. A trypsin (IU/l) : elastase (IU/l) inhibitory ratio of 0.3 was calculated for α_1-PI in fraction 15 and of 5.0 for ITI in fraction 26.
b) Rocket immunoelectrophoresis of the indicated fractions. For identification either 1% ITI antiserum or 1.5% α_1-PI antiserum was used. Precipitin lines were not caused by any other eluted fractions.
For experimental details, see Material and Methods.

for ITI and α_1-PI, respectively, the prepurified substances were chromatographed separately. As shown in the elution diagram (Fig. 1a) established by the inhibitory activities of the fractionated samples against trypsin and granulocytic elastase, ITI* was eluted in fractions 24 to 28 but α_1-PI already in fractions 13 to 18. This was also confirmed by rocket immunoelectrophoresis using specific ITI and α_1-PI antiserum, respectively (Fig. 1b).

In a first approach, fractionation of 1 m*l* human serum was performed with a step-wise NaCl gradient ranging from 0 to 1.0M. Under these conditions two peak areas each with inhibitory activity towards trypsin and granulocytic elastase could be separated (Fig. 2a). According to the inhibitory elution pattern of the prepurified inhibitors (Fig. 1a) as confirmed by the results of rocket immunoelectrophoresis (Fig. 2b), fractions 24 to 27 contained ITI and fractions 9 to 16 α_1-PI. Molecular mass estimation by dodecyl sulfate gel electrophoresis under reducing conditions showed for the protein(s) in fraction 25 and 26 a mean molecular mass of 178 kDa (Fig. 3). This is in good agreement with the value of 182 kDa found for the protein in comparable fractions after chromatography of prepurified ITI (Fig. 3). The presence of only trace amounts of proteins smaller than ITI in fraction 25 and 26, indicates a high degree of purification of ITI by this single chromatographic step. Proteins present in fractions 10 to 14 after ion-exchange chromatography of human serum have a mean molecular mass of 60 kDa; they probably present albumin and α_1-PI.

In a second approach higher quantities of ITI could be isolated from 5 m*l* serum without overloading the ion exchanger. To achieve this, a linear NaCl gradient was started already with 0.3M saturation. Under these conditions, α_1-PI (identified by the inhibition spectrum and the given immunological assays) was eluted with the bulk of the proteins in the non-retarded fractions. ITI was eluted in several fractions identified by their inhibitory activity towards trypsin and

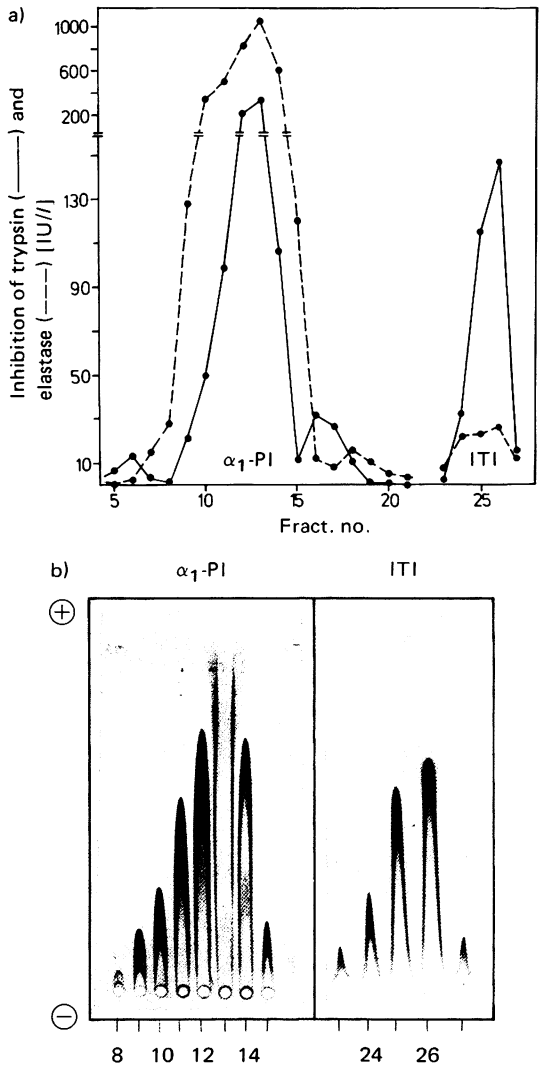


Fig. 2. Separation of 1 m*l* human serum with the FPLC System.

a) The elution diagram represents the inhibitory activities of each fraction towards trypsin (•——•) and granulocytic elastase (•– – –•). A step-wise NaCl gradient was applied ranging from 0–0.2M (fractions 3 to 17), 0.2–0.4M (fractions 17 to 24), and 0.4–1.0M (fractions 24 to 28).

A trypsin (IU/*l*): elastase (IU/*l*) inhibitory ratio of 0.3 was calculated for α_1-PI in fraction 13 and of 5.5 for ITI in fraction 26.

b) Rocket immunoelectrophoresis of the indicated fractions. For identification either 1% ITI antiserum or 1.5% α_1-PI antiserum was used. Precipitin lines were not caused by any other eluted fractions.

* A detailed description of the separation method for the "prepurified" ITI will be published elsewhere (Karges, H., Angerer, F., Jochum, M., in preparation).

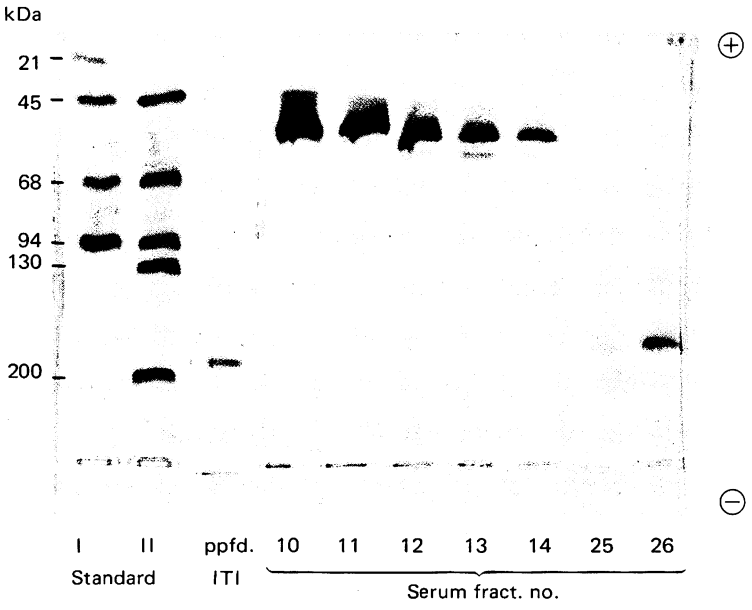


Fig. 3. Dodecyl sulfate polyacrylamide gel (7.5%; 1% 2-mercaptoethanol) electrophoresis of selected fractions after chromatography of prepurified ITI (fraction 26; ppfd. ITI) or serum with the FPLC System.
Standard I (from ⊖ to ⊕): Phosphorylase B (M_r 94000), bovine serum albumin (M_r 68000), ovalbumin (M_r 45000), soybean trypsin inhibitor (M_r 21000), lysozyme (M_r 14300).
Standard II (from ⊖ to ⊕): Myosin (M_r 200000), β-galactosidase (M_r 130000), phosphorylase B (M_r 94000), bovin serum albumin (M_r 68000), ovalbumin (M_r 45000).
For experimental details, see Materials and Methods.

granulocytic elastase (Fig. 4a), as well as the immunological reaction with ITI-specific antibodies (Fig. 4b).

The results given here show clearly that in human serum, in addition to α_1-PI and α_2-macroglobulin[13], a further high molecular mass protein is able to inhibit granulocytic elastase. This protein is obviously identical with ITI isolated by Hochstraßer and coworkers, although these authors failed to demonstrate granulocytic elastase inhibition by their various inhibitor preparations up to now[2,7]*. Hence, ion-exchange chromatography using the FPLC System can be recommended as a convenient method for rapid isolation of the labile, inhibitorily fully active ITI from human serum.

Discrimination of ITI from α_1-PI is also possible on the basis of the ratio of their inhibitory activities towards trypsin and elastase. Using appropriate inhibitory activity (IU/*l*) of the eluted main peak fractions, a trypsin : elastase inhibitory ratio of 0.3 was calculated for α_1-PI and of about 5 for ITI.

This work was supported by the *Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 0207* (LP8).
We are very grateful to Mr *Blaicher* and Mr *Häringer*, Deutsche Pharmacia, Freiburg/Br. for supplying us with the FPLC System.
We also wish to thank Dr. H. Karges, Behringwerke Marburg, for ITI and ITI antiserum as well as Dr. Gunzer, E. Merck, Darmstadt, for α_1-PI.

* Granulocytic elastase inhibition by ITI and its acid-stable inhibitory derivative, respectively, was recently observed by Albrecht, G.J., Hochstraßer, K. and Salier, J.-P. (1983) *Hoppe-Seyler's Z. Physiol. Chem.* **364**, 1703–1708, preceding paper.

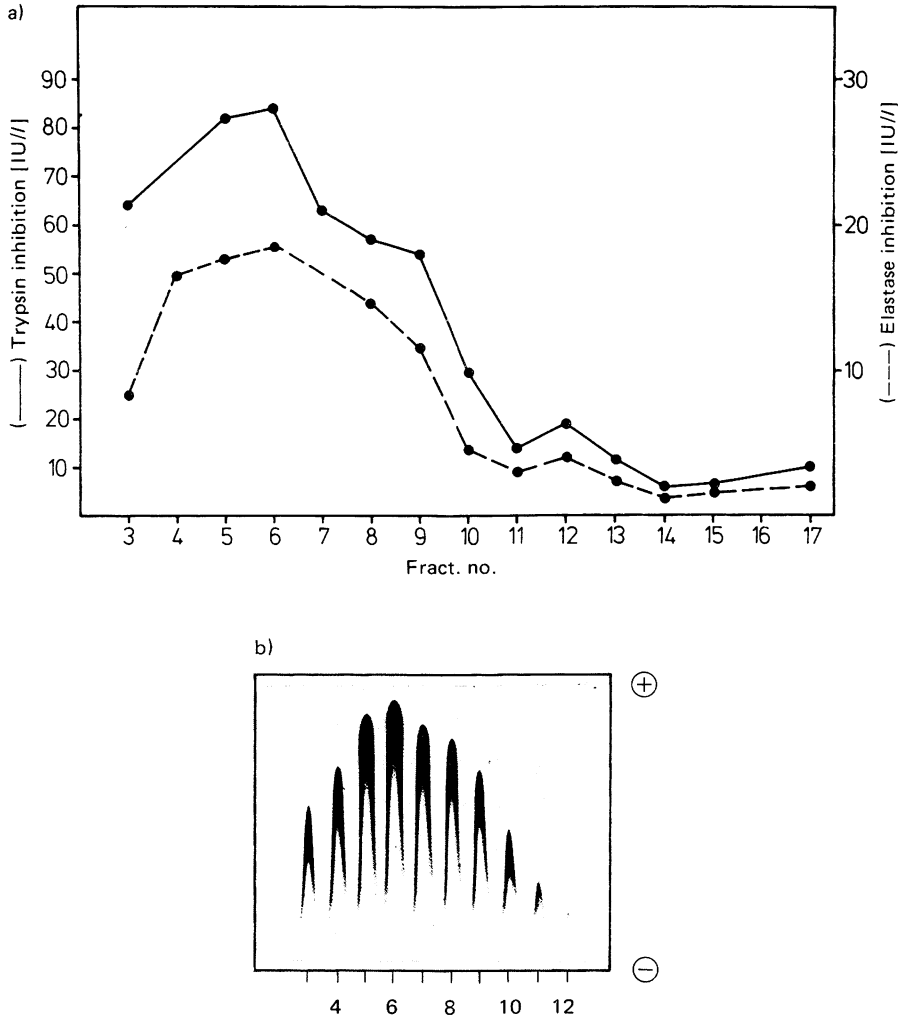


Fig. 4. Separation of 5 m*l* human serum with the FPLC System.

a) The elution diagram represents the inhibitory activities of each fraction towards trypsin (●——●) and granulocytic elastase (●– – –●).

A linear NaCl gradient was applied from 0.3M to 1.0M.

A trypsin (IU/*l*) : elastase (IU/*l*) inhibitory ratio of 5.0 was calculated for ITI in fraction 6.

b) Rocket immunoelectrophoresis of the indicated fractions. For identification 1% ITI antiserum was used. Precipitin lines were not caused by any other eluted fractions.

For experimental details, see Material and Methods.

Literature

1 Steinbuch, M. (1976) *Methods Enzymol.* **45**, 760–772.
2 Dietl, T., Dobrinski, W. & Hochstraßer, K. (1979) *Hoppe-Seyler's Z. Physiol. Chem.* **360**, 1313–1318.
3 Bieth, J., Aubry, M. & Travis, J. (1973) in *Proteinase Inhibitors, Proc. 2nd. Int. Res. Conf. (Bayer Symp. V)*, (Fritz, H., Tschesche, H., Greene, L.J. & Truscheit, E., eds.), pp. 53–62, Springer-Verlag, Berlin.
4 Fritz, H., Heimburger, N., Meier, M., Arnhold, M., Zaneveld, L. & Schumacher, G. (1972) *Hoppe-Seyler's Z. Physiol. Chem.* **353**, 1953–1956.
5 Jönsson, B.M., Löffler, Ch. & Ohlsson, K. (1982) *Hoppe-Seyler's Z. Physiol. Chem.* **363**, 1167–1175.
6 Bromke, B.J. & Kueppers, F. (1982) *Biochem. Med.* **27**, 56–67.
7 Hochstraßer, K., Bretzel, G., Feuth, H., Hilla, W. & Lempart, K. (1976) *Hoppe-Seyler's Z. Physiol. Chem.* **357**, 153–162.
8 Baugh, R.J. & Travis, J. (1976) *Biochemistry* **15**, 836–841.
9 Wenzel, H.R., Engelbrecht, S., Reich, H., Mondry, W. & Tschesche, H. (1980) *Hoppe-Seyler's Z. Physiol. Chem.* **361**, 1413–1416.
10 Fritz, H., Trautschold, I. & Werle, E. (1970) in *Methoden der Enzymatischen Analyse* (Bergmeyer, H.U., ed.) 2nd edn., pp. 1021–1031, Verlag Chemie, Weinheim/Bergstraße.
11 Laurell, C.-B. (1972) *Scand. J. Clin. Lab. Invest.* **29**, *Suppl.* **124**, 21–37.
12 Salier, J.P., Lambin, P., McPhee, H. & Hochstraßer, K. (1980) *Anal. Biochem.* **109**, 273–283.
13 Ohlsson, K., Olsson, I. (1974) *Scand. J. Clin. Lab. Invest.* **34**, 349–355.

Dr. Marianne Jochum and Astrid Bittner, Abteilung für Klinische Chemie und Klinische Biochemie in der Chirurgischen Klinik Innenstadt der Universität München,
D-8000 München 2, Nußbaumstraße 20.

Autorenverzeichnis

Die mit * versehenen Seitenzahlen beziehen sich auf Kurzreferate von Tagungen und Konferenzen