fluorescence. No antibody response is found in the midpiece and head of the epididymis and testes tissue. The seminal plasma inhibitors are mainly produced in the seminal vesicles. No immunological cross-reaction was observed with the inhibitors present in the epididymis and testes.

The indirect immunofluorescence antibody technique revealed also that ejaculated spermatozoa carry seminal inhibitors, probably at the outer acrosomal region, that the inhibitors are removed during residence (capacitation) in the uterine fluid (6 hr), and that uterine spermatozoa can be recharged with seminal inhibitors.

The inhibition of *in vivo* fertilization by treatment of capacitated spermatozoa by boar seminal inhibitors has been reported.[4,6]

### Acknowledgments

This work was supported by Sonderforschungsbereich 51, Munich, and WHO Geneva (Grants Nos. 2837 and 74,053). We wish to thank S. Kupfer and M. Meier for skillful technical assistance as far as unpublished work is concerned.

## [75] Acid-Stable Proteinase Inhibitors from Human Seminal Plasma

*By* HANS SCHIESSLER, EDWIN FINK, and HANS FRITZ

Antitryptic activity in human seminal plasma was first detected by Rasmussen and Albrechtson.[1] Haendle *et al.*[2] described the occurrence of acid-stable trypsin inhibitors in testes, epididymis, and seminal vesicles as well as in the seminal plasma of many mammals, including man.

Fink *et al.*[3] and Suominen and Niemi[4] showed that the antitryptic activity in human seminal plasma is due to two different trypsin inhibitors, the human seminal plasma inhibitors (HUSI) I and II. HUSI-I, a trypsin–chymotrypsin inhibitor, and HUSI-II, a trypsin-acrosin inhibitor, were further characterized in our laboratory.[5,6] $\alpha_1$-Antitrypsin, an

[1] J. Rasmussen and O. K. Albrechtson, Fertil. *Steril.* **11**, 264 (1960).
[2] H. Haendle, H. Fritz, I. Trautschold, and E. Werle, *Hoppe-Seyler's Z. Physiol. Chem.* **343**, 185 (1965).
[3] E. Fink, E. Jaumann, H. Fritz, H. Ingrisch, and E. Werle, *Hoppe-Seyler's Z. Physiol. Chem.* **352**, 1591 (1971).
[4] J. Suominen and M. Niemi, *J. Reprod. Fertil.* **29**, 163 (1972).
[5] H. Schiessler, M. Arnhold, and H. Fritz, *Proteinase Inhibitors, Proc. Int. Res, Conf., 2nd, (Bayer Symp. V)*, Grosse Ledder, 1973 p. 147. Springer-Verlag, Berlin and New York, 1974.
[6] H. Schiessler, M. Arnhold, and H. Tschesche, *Abstr. Annu. Meet. Soc. Study Reprod.*, 1974, p. 79.

acid-unstable glycoprotein, is also present in human seminal plasma.[7]

Trypsin inhibitors isolated from human spermatozoa[5,6,8-10] show very similar characteristics to those of HUSI-I and HUSI-II.

## Assay Methods

### *Trypsin Inhibition*

*Principle.* The inhibition of the tryptic hydrolysis of $N^{\alpha}$-benzoyl-DL-arginine *p*-nitroanilide (DL-BAPA) is measured by following the change in absorbance at 405 nm. This standard assay for the determination of the inhibitory activity has been described by Fritz *et al.*[11] and also by Kassell[12] in this series.

However, $N^{\alpha}$-benzoyl-L-arginine *p*-nitroanilide (L-BAPA) is a better substrate than DL-BAPA in this test system,[13] since the D-isomer is a competitive inhibitor of trypsin. L-BAPA has the further advantage of greater solubility and a higher turnover number with trypsin.

### *Chymotrypsin Inhibition*[11]

*Principle.* The inhibition of the chymotrypsin-catalyzed release of *p*-nitroaniline from $N^{\alpha}$-succinyl-L-phenylalanine *p*-nitroanilide (SUPHEPA) is measured by following the change in absorbance at 405 nm.

#### *Reagents*

Substrate: 250 mg of SUPHEPA dissolved in 50 ml of buffer

Buffer: 0.2 *M* triethanolamine (TRA), pH 7.8, containing 0.02 *M* $CaCl_2$

Enzyme: crystalline $N^{\alpha}$-*p*-toluenesulfonyl-L-lysyl chloromethyl ketone (TLCK)-treated chymotrypsin is dissolved in 0.0025 *N* HCl at a concentration of 2 mg/10 ml. The solution is stored at 4°.

[7] G. F. B. Schumacher, *Proteinase Inhibitors, Proc. Int. Res. Conf., 1st,* Munich, 1970 p. 245. de Gruyter, Berlin, 1971.

[8] H. Fritz, B. Förg-Brey, E. Fink, M. Meier, H. Schiessler, and C. Schirren, *Hoppe-Seyler's Z. Physiol. Chem.* **353**, 1943 (1972).

[9] L. J. D. Zaneveld, B. M. Dragoje, and G. F. B. Schumacher, *Science* **177**, 702 (1972).

[10] F. N. Syner and R. Kuras, *Abstr. Annu. Meet. Soc. Study Reprod.*, 1974, p. 108.

[11] H. Fritz, I. Trautschold, and E. Werle, *in* "Methoden der Enzymatischen Analyse" (H. U. Bergmeyer, ed.), 2nd ed., Vol. I, p. 1021. Verlag Chemie, Weinheim, 1970.

[12] B. Kassell, this series Vol. 19, p. 845.

[13] H. Naketa and S. Ishi, *J. Biochem.* **72**, 281 (1972).

*Procedure.* The mixture of 0.1 ml of the enzyme solution (corresponding to 6.7 μg of chymotrypsin per milliliter), the inhibitor solution and the buffer (final volume 2.0 ml) is preincubated for 5 min. Preincubation and the kinetic test are performed at a temperature of 25°. The enzymic reaction is started by addition of 1.0 ml of the substrate solution to the preincubation mixture. The increase in absorption at 405 nm is followed for 20 min. The chymotrypsin activity of the inhibitor-free control serves as a reference value for the calculation of the inhibitory activity.

For the determination of very low inhibitor concentrations or kinetic data, the following substrates are recommended: $N^{\alpha}$-benzoyl-L-arginine ethyl ester (BAEE) and $N^{\alpha}$-acetyl-L-tyrosine ethyl ester (ATEE). The conditions to be used for the trypsin-catalyzed hydrolysis of BAEE and the chymotrypsin-catalyzed hydrolysis of ATEE are given by Schwert and Takenaka.[14] The assay of trypsin or chymotrypsin inhibition with these substrates is performed in identical test systems. The trypsin-inhibition test with BAEE is extensively described by Burck[15] in this series.

### *Acrosin Inhibition*

*Principle.* The substrate specificities of acrosin and trypsin are very similar.[16] Spectrophotometric measurement of the rate of acrosin-catalyzed hydrolysis of BAEE, DL-BAPA, L-BAPA, or $N^{\alpha}$-benzoyl-L-lysine *p*-nitroanilide (L-BLNA) is suitable for quantitative determination of acrosin.[17] Therefore, the acrosin-inhibition assay may be performed using test systems employed for trypsin inhibition.[18-21] However, purified acrosin is strongly adsorbed and inactivated on glass surfaces; the use of plastic cuvettes is recommended to minimize this effect.[22]

[14] G. W. Schwert and Y. Takenaka, *Biochim. Biophys. Acta* **16,** 570 (1955).
[15] P. J. Burck, this series Vol. 19, p. 907.
[16] H. Schiessler, W.-D. Schleuning, and H. Fritz, *Hoppe-Seyler's Z. Physiol. Chem.* **356,** 1931 (1975).
[17] W.-D. Schleuning and H. Fritz, this volume [27].
[18] H. Fritz, B. Förg-Brey, E. Fink, H. Schiessler, E. Jaumann, and M. Arnhold, *Hoppe-Seyler's Z. Physiol. Chem.* **353,** 1007 (1972).
[19] H. Schiessler, H. Fritz, M. Arnhold, E. Fink, and H. Tschesche, *Hoppe-Seyler's Z. Physiol. Chem.* **353,** 1638 (1972).
[20] H. Fritz, B. Förg-Brey, M. Meier, M. Arnhold, and H. Tschesche, *Hoppe-Seyler's Z. Physiol. Chem.* **353,** 1950 (1972).
[21] L. J. D. Zaneveld, G. F. B. Schumacher, H. Fritz, E. Fink, and E. Jaumann, *J. Reprod. Fertil.* **32,** (1973).
[22] E. Fink, H. Schiessler, M. Arnhold, and H. Fritz, *Hoppe-Seyler's Z. Physiol. Chem.* **353,** 1633 (1972).

*Definition of Units and Specific Activity.* One enzyme unit corresponds to the hydrolysis of 1 μmole of substrate per minute under the given conditions. The change in absorbance due to activity $A_{405}^{1\,cm}$/min = 3.32 for the substrates BAPA, BLNA, and SUPHEPA and $A_{253}^{1\,cm}$/min = 0.385 for BAEE. One inhibitor unit causes the reduction of the enzyme activity by one enzyme unit. The specific activity is expressed in inhibitor units per milligram of protein.

*Inhibition of Leukocytic Proteinases*

*Principle.* Azocasein is digested by neutral proteinases from leukocytes to products that are soluble in trichloroacetic acid (TCA). The activity of the proteinases is measured by following the increase in absorbance at 366 nm in the TCA solution (due to the split products). Inhibition is calculated from the difference between the absorbance of the inhibitor-containing samples and the control.

*Reagents*

Substrate: 2 g of azocasein dissolved in 100 ml of warm buffer

Buffer: 0.1 *M* $Na_2HPO_4$, pH 7.65

Enzyme: Proteinases from human leukocytes. The enzyme preparation is extracted from leukocytes of maxillopharyngeal abscesses according to Hochstrasser *et al.*[23] Of the enzyme-containing powder, 75 mg are dissolved in 5 ml of buffer. Insoluble material is removed by centrifugation at 4°. In the test system used, 1.12–4.5 mg of the enzyme preparation caused an increase in absorbance $A_{366}^{1\,cm}$ from $0.28 \times 10^{-3}$ to $1.2 \times 10^{-3}$ per minute.

Inhibitor solution: About 0.1 mg of inhibitor is dissolved in 1.0 ml of buffer.

TCA: 5 g of TCA are dissolved in 5 ml of distilled water.

*Procedure.* Constant amounts of the leukocytic enzyme preparation, 4.5 mg in 0.3 ml buffer, are incubated at 25° for 1 hr with increasing amounts of inhibitor in the phosphate buffer, pH 7.65, in a final volume of 1 ml. The enzyme reaction is started by addition of 2.0 ml substrate solution to each of the preincubation mixtures. After 3 hr reaction time at 37°, proteolysis is stopped by addition of 3.0 ml of TCA solution. The absorbance of the centrifuged and filtered supernatant is measured at 366 nm.

The values obtained were corrected by subtraction of the absorbance of a reference blank containing all reagents but incubated for 0 min.

[23] K. Hochstrasser, R. Reichert, S. Schwartz, and E. Werle, *Hoppe-Seyler's Z. Physiol. Chem.* 353, 221 (1972).

TABLE I
SUMMARY OF THE PURIFICATION PROCEDURE

| | Step and fraction | Total inhibitory activity[a] (IU) | Yield (%) | Specific activity[a] (IU/mg) |
|---|---|---|---|---|
| | Sperm plasma, 1 liter | 120 | (100) | 0.00009 |
| 1 | SP-Sephadex C-50 | 108 | 90 | 0.017 |
| 2 | Trypsin cellulose | 92 | 78 | 1.2 |
| 3 | Sephadex G-75 | 82.5 | 69 | |
| | HUSI[c]-I | 38.3 | 32 | 1.37 |
| | HUSI-II | 44.2 | 37 | 1.3 |
| 4 | SP-Sephadex C-25 | | | |
| | HUSI-I | 34.5 | 90[b] | 1.6 |
| | HUSI-II | 40 | 90[b] | 2.22 |

[a] Trypsin inhibition, substrate: $N^{\alpha}$-benzoyl-DL-arginine *p*-nitroanilide.
[b] Referred to the corresponding preparation from step 3.
[c] HUSI, human seminal plasma inhibitor.

## Purification Procedure

The procedure is summarized in Table I.

### *Starting Material*

A trypsin inhibitor activity of 150–330 mIU/ml is detectable in freshly ejaculated human seminal plasma.[24] This corresponds to the inhibition of about 0.1–0.3 mg of bovine trypsin. We received most of our material from andrology clinics. These ejaculates had been frozen and stored up to one year. The inhibitor concentration in several of these batches was up to 50% less than in freshly collected ejaculates.[25]

Degradation of the inhibitors by proteinases of seminal plasma and spermatozoa may be responsible of this reduction. The average inhibitor concentrations of abnormal ejaculates (oligospermia, azoospermia, aspermia, hypocinetic spermatozoa) and normal ejaculates are not significantly different. The seminal plasma of such ejaculates is also suitable for inhibitor isolation.

### *Storage Conditions*

In order to prevent extensive degradation, it is recommended that the spermatozoa be separated from the seminal plasma by centrifugation

[24] H. Haendle, Dissertation, Medical Faculty, University of Munich, 1969.
[25] C. Buck, *Andrologie* **5**, 23 (1973).

(600 *g*, 20 min, 4°) immediately after liquefaction of the ejaculates. Spermatozoa-free plasma should be frozen preferably below —70°. After thawing, the inhibitors should be separated from the residual seminal fluid without delay.

*Step 1. Chromatography on SP-Sephadex C-50*

This step should be performed rapidly and under constant cooling (4°) after thawing the frozen ejaculates or seminal plasma.

*Reagents*

Ammonium acetate buffer, 0.05 *M*, pH 5.4; 15 liters containing 0.02% (w/v) $NaN_3$

SP-Sephadex C-50, 30 g; i.e., 1 liter of resin, swollen and equilibrated with buffer

NaCl, 292 g

*Procedure.* One liter of a frozen ejaculate pool is thawed (4°) and centrifuged (600 *g*) for 2 hr at 4°. The supernatant is removed and tested for inhibitory activity. The sediment serves for the isolation of the inhibitors from spermatozoa and of acrosin.

The high-molecular-weight, acid-labile proteins have to be denatured by acidification of the seminal plasma before the concentration of the acid-stable inhibitors can be estimated.

Seminal plasma, 0.5 ml, is diluted with the same volume of perchloric acid (6% w/v). Denatured proteins are removed by centrifugation (6000 *g*, 15 min, 4°). The clear supernatant is neutralized by addition of solid potassium hydrogen carbonate. If solutions of high ionic strength are applied to the preincubation mixture (enzyme plus inhibitor plus buffer), inhibitor-free controls of the same ionic strength should be employed in order to avoid misleading results.

The seminal plasma is diluted with the same volume of buffer. The mixture is adjusted to pH 5.4 by addition of acetic acid. Thirty grams of SP-Sephadex C-50, equilibrated with the buffer solution, are added and the suspension is stirred slowly for 2 hr at 4°. After centrifugation (600 *g*, 15 min, 4°) the resin-free supernatant is decanted and the adsorbent is washed three times with 1 liter of buffer solution. The adsorbent is then packed into a $7 \times 50$ cm column. The column is washed with the buffer solution at a rate of 140 ml/hr until the transmission of the eluate has dropped to that of the buffer solution. Then, the column is developed with a linear sodium ion gradient formed from 2 liters of

starting buffer (0.05 *M* ammonium acetate buffer pH 5.4) and 2 liters of the same buffer containing 1 *M* sodium chloride.

The inhibitors are eluted in a single peak between 0.15 *M* and 0.5 *M* sodium ion concentration. The combined inhibitor fractions are neutralized with 2 *N* NaOH and concentrated by evaporation using a rotary evaporator (water bath temperature, 30°). Desalting is performed by passing the concentrated inhibitor solution (max. 25 ml) through a Sephadex G-25 column (120 × 3.6 cm) equilibrated and developed with 2% (v/v) acetic acid. The salt-free inhibitor fractions are combined and lyophilized. Specific activities between 19 and 25 mIU/mg (trypsin inhibition; substrate, DL-BAPA) were estimated for the material thus obtained.

*Step 2. Affinity Chromatography*[26,27]

*Reagents*

A. "Trypsin, polymer bound on CM-Cellulose" purchased from E. Merck, Darmstadt (article No. 24,582, capacity 7–10 U/mg). One gram of the trypsin cellulose is equilibrated with buffer B. Fine particles are removed. The trypsin cellulose is poured into a cooled (0°) column (1.2 × 10 cm) and washed with buffer B until trypsin and other proteins are no longer detectable in the eluate. Used trypsin cellulose may be stored in acidic buffer (C) at 4°.

B. Washing buffer: 0.1 *M* triethanolamine-HCl, 0.4 *M* NaCl, pH 7.8

C. Acidic buffer: 0.4 *M* KCl-HCl, pH 1.8

*Procedure.* The dry material from step 1 is dissolved in 50 ml of buffer B. The inhibitor solution is applied to the column, running at a flow rate of 16 ml/hr. The effluent is collected until excess inhibitor appears in the eluate. Then the column is washed with buffer B until the effluent is entirely protein free. Dissociation of the inhibitors from the trypsin–inhibitor complex, and thus their elution from the column, is achieved by applying buffer C. Elution is stopped when the effluent is free of inhibitor. After this procedure the trypsin-cellulose column has to be recquilibrated with buffer B.

Batchwise operation is also possible. The trypsin–cellulose is carefully suspended in the inhibitor solution for 1 hr (4°). The adsorbent is

[26] B. Kassell, this series Vol. 19, p. 846.

[27] H. Fritz, B. Brey, M. Müller, and M. Gebhardt, *Proteinase Inhibitors, Proc. Int. Res. Conf. 1st,* Munich, 1970 p. 28. de Gruyter, Berlin, 1971.

then washed 6 times each with 50 ml of buffer B by repeated suspension (for 5 min) and centrifugation. Elution of the inhibitors is achieved by repeated (6 times) suspension of the adsorbent in 50 ml of buffer C each time. The degree of purification and yields are comparable for both methods.

The acidic inhibitor fractions are combined and concentrated by ultrafiltration at 4° to a final volume of 10 ml in an Amicon cell equipped with an UM-05 membrane.

*Step 3. Separation of HUSI-I and HUSI-II by Gel Filtration on Sephadex G-75*

Separation of the antitryptic activity into two fractions, a trypsin–chymotrypsin inhibitor, HUSI-I, and a trypsin–acrosin inhibitor, HUSI-II, is achieved by gel filtration on Sephadex G-75.

Concentrated inhibitor solution from step 2, 5 ml, is applied to a water-cooled (10°) Sephadex G-75 column (128 × 1.8 cm) equilibrated and developed with 2% (v/v) acetic acid at a flow rate of 16 ml/hr. Fractions of 5 ml are collected. After elution of impurities at the void volume, the trypsin-inhibiting activity appears separated into two fractions. The first fraction ($v/v_0 = 1.84$) corresponding to HUSI-I is followed by the HUSI-II fraction ($v/v_0 = 2.23$). The inhibitory activity should be estimated in each tube between 40 and 80. The contents of the tubes containing HUSI-I and HUSI-II are pooled separately and lyophilized. Tubes in the overlap region (Nos. 57–61) containing both inhibitors have to be rechromatographed in the same system.

If the column is calibrated with reference proteins, estimation of the molecular weights of the inhibitors is possible.

*Step 4. Chromatography on SP-Sephadex C-25*

Both HUSI-I and HUSI-II from step 3 are submitted to gradient elution chromatography using identical conditions.

*Reagents*

SP-Sephadex C-25 (Pharmacia Fine Chemicals)
Buffer I: 0.05 *M* $Na_2HPO_4$, pH 6.12
Buffer II: 0.05 *M* $Na_2HPO_4$, pH 6.12, 0.4 *M* NaCl ($Na^+ = 0.5$ *M*)

*Procedure.* HUSI-I or HUSI-II, 50 mg, is dissolved in 2–3 ml of buffer I. The clear solution is applied to the SP-Sephadex C-25 column (120 × 1 cm) equilibrated with buffer I. The column is developed with a linear sodium ion gradient formed from 0.8 liter each of buffer I and buffer II at a flow rate of 7 ml/hr; 3 ml fractions are collected.

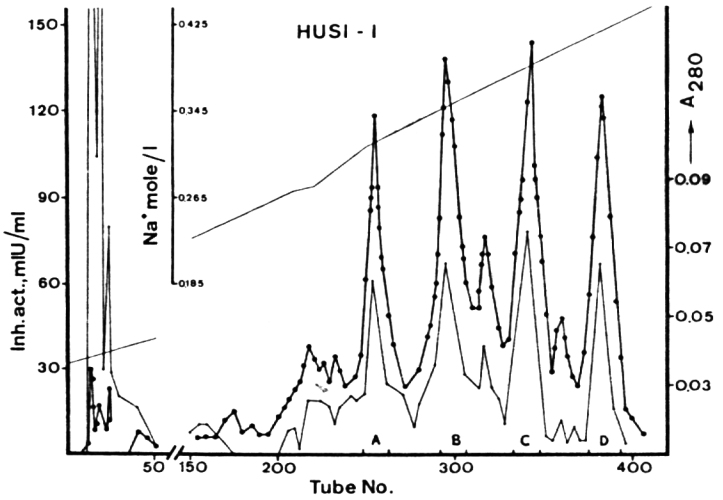


FIG. 1. Fractionation of human seminal plasma inhibitor (HUSI) I by gradient elution chromatography on SP-Sephadex C-25 HUSI-I separated from HUSI-II by gel filtration (step 3) was employed. The column (120 × 1 cm) was equilibrated with sodium phosphate buffer ($Na^+$ = 0.1 *M*), pH 6.12, and developed with a linear sodium ion gradient formed from 0.8 liter each of starting buffer and 0.4 *M* NaCl. Flow rate: 7 ml/hr, 3 ml/fraction. See text, step 4.

Chromatography of the inhibitors HUSI-I and HUSI-II results in the separation of each inhibitor fraction into 4 multiple forms (cf. Figs. 1 and 2). The sodium ion concentration, by which the varous inhibitor fractions appear in the eluate, as well as the distribution of the inhibitory activity among the fractions, are given in Table II. Tubes containing the same inhibitor fraction are combined, concentrated by evaporation, and desalted by gel filtration on a Sephadex G-25 column (70 × 2 cm) equilibrated and developed with 2% (v/v) acetic acid. After lyophilization of the salt-free inhibitor fractions, each individual form may be subjected to equilibrium chromatography.

*Step 5. Final Purification by Equilibrium Chromatography*

The final purification of each individual fraction may be carried out by equilibrium chromatography on a SP-Sephadex C-25 column (120 × 1 cm; flow rate 7 ml/hr; 3 ml/fraction) equilibrated and developed with a phosphate buffer of constant sodium ion concentration. The exact concentration used corresponds to that at which the applied inhibitor is eluted during the fractionation in step 4 (cf. Table II). Desalting is performed as in step 4.

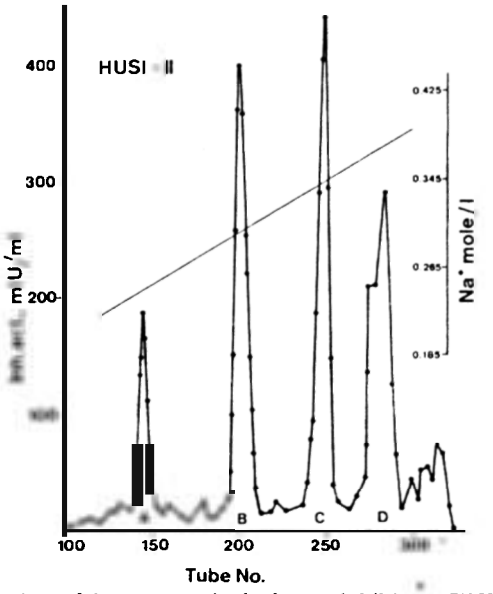


FIG. 2. Fractionation of human seminal plasma inhibitor (HUSI) II by gradient elution chromatography on SP-Sephadex C-25. HUSI-II obtained by gel filtration on Sephadex G-75 (step 3) was employed. The conditions of this chromatographic step are identical to those mentioned in Fig. 1. See text, step 4.

TABLE II

SEPARATION OF THE MULTIPLE FORMS OF HUMAN SEMINAL PLASMA INHIBITOR (HUSI) I AND II FROM STEP 3 BY GRADIENT ELUTION CHROMATOGRAPHY ON SP-SEPHADEX C-25[a]

| | HUSI-I fraction | | | |
|---|---|---|---|---|
| | A | B | C | D |
| $Na^+$ (moles/liter) | 0.305 | 0.34 | 0.38 | 0.41 |
| Inhibitory activity (%)[b] | 15 | 30 | 24 | 31 |
| | HUSI-II fraction | | | |
| | A | B | C | D |
| $Na^+$ (moles/liter) | 0.21 | 0.28 | 0.335 | 0.365 |
| Inhibitory activity (%)[b] | 9 | 26 | 24 | 41 |

[a] The multiple forms A to D of both inhibitors HUSI-I and HUSI-II appear in the eluate at sodium ion concentrations shown in the table. Rechromatography of each fraction is performed using these same sodium ion concentrations.

[b] Related to the total inhibitory activity eluted.

## Properties

*Stability.* Both inhibitors are stable in solutions of pH 2-9 at room temperature. Some lyophilized batches of HUSI-II (step 3) showed a reduction of the specific activity after prolonged storage at 4°.

*Molecular Weight.* Molecular weight values between 11,000 and 14,500 were found for HUSI-I and 4000 to 6500 for HUSI-II by gel filtration experiments.[3-5] From the amino acid compositions shown in Table III, a molecular weight near 10,500 is calculated for HUSI-I fraction C and 6217 for HUSI-II fraction D.

*Amino Acid Composition.* Table III shows the amino acid compositions of HUSI-I fraction D and C and HUSI-II fraction D. Compared

TABLE III
AMINO ACID COMPOSITION OF ACID-STABLE PROTEINASE INHIBITORS FROM HUMAN SEMINAL PLASMA[a]

| | Amino acid residues/molecule | | |
|---|---|---|---|
| | HUSI[b]-I | | HUSI-II |
| Amino acid | D | C | D |
| Aspartic acid | 8 | 8 | 6 |
| Threonine | 4 | 4 | 3 |
| Serine | 6 | 6 | 4 |
| Glutamic acid | 7 | 8 | 3 |
| Proline | 12 | 12 | 5 |
| Glycine | 9 | 9 | 5 |
| Alanine | 3 | 3 | 1 |
| Half-cysteine | 12 | 12 | 6 |
| Valine | 5 | 5 | 1 |
| Methionine | 3 | 3 | 1 |
| Isoleucine | 1 | 1 | 3 |
| Leucine | 4 | 5 | 2 |
| Tyrosine | 2 | 2 | 3 |
| Phenylalanine | 2 | 2 | 1 |
| Lysine | 12 | 12 | 3 |
| Histidine | 0 | 1 | 2 |
| Arginine | 4 | 4 | 5 |
| Tryptophan | 0 | 0 | 0 |
| | 94 | 97 | 54 |
| Molecular weight | 10,130 | 10,510 | 6,217 |

[a] The molecular weights given were calculated from the amino acid compositions. Amino sugars were not detectable.

[b] HUSI, human seminal plasma inhibitor.

TABLE IV
INHIBITION SPECIFICITY OF HUMAN SEMINAL PLASMA INHIBITOR (HUSI) I AND II

| Proteinases[a] | Substrate[b] | HUSI-I | HUSI-II |
|---|---|---|---|
| Trypsin, | | | |
| Human, cationic[c] | TAME | + | + |
| Human, anionic[c] | TAME | + | + |
| Bovine[3-5] | BAEE, DL-BAPA | + | ++ |
| Acrosin, | | | |
| Human[5,10,20,21] | BAEE, DL-BAPA | − | ++ |
| Boar[5,18] | DL-BAPA | − | ++ |
| Chymotrypsin, bovine[3-5] | ATEE, SUPHEPA | ++ | |
| Leukocytic proteinases, human, neutral[5] | Azocasein | ++ | − |
| Elastase, | | | |
| Human, granulocytic[d] | Elastine | + | − |
| Porcine, pancreatic | Elastine | − | − |
| Chymotrypsin-like proteinase from human sperm plasma[e] | ATEE, azocasein | − | − |

[a] Superscript numbers refer to text footnotes.

[b] TAME, $N^{\alpha}$-toluenesulfonyl-L arginine methyl ester; BAEE, $N^{\alpha}$-benzoyl-L-arginine ethyl ester; DL-BAPA, $N^{\alpha}$-benzoyl-DL-arginine *p*-nitroanilide; ATEE, $N^{\alpha}$-acetyl-L-tyrosine ethyl ester; SUPHEPA, $N^{\alpha}$-succinyl-L-phenylalanine *p*-nitroanilide.

[c] C. Figarella, G. A. Negri, and O. Guy, *Proteinase Inhibitors, Proc. Int. Res. Conf., 2nd, (Bayer Symp. V)*, Grosse Ledder, 1973 p. 213. Springer-Verlag, Berlin and New York, 1974.

[d] K. Ohlsson, personal communications.

[e] H. Fritz, M. Arnhold, B. Förg-Brey, L. J. D. Zaneveld, and G. F. B. Schumacher, *Hoppe-Seyler's Z. Physiol. Chem.* **353**, 1651 (1972).

to HUSI-I fraction D, HUSI-I fraction C contains three additional amino acid residues, whereas the compositions of fraction A and B are identical with that of fraction D. Both inhibitors contain neither tryptophan nor amino sugar residues.

The amino acid compositions of inhibitors isolated from freshly collected ejaculates may show differences from the compositions given in Table III. Peptidases present in the ejaculate may cause loss of amino acids or peptides by partial proteolytic degradation.

*N-Terminal Residues.* The only N-terminal residue for HUSI-I fraction D found by substractive Edman degradation[28] was tyrosine followed by leucine.

*Inhibition Specificity* (cf. Table IV). Neither of the two inhibitors

[28] M. Percy and B. Buchwald, *Anal. Biochem.* **45**, 60 (1972).

has any effect on the enzyme activity of human plasmin (Deutsche Kabi GmbH) and porcine plasmin (Novo Industri A/S), porcine pancreatic kallikrein,[29] subtilisin (Serva AG), *Aspergillus oryzae* proteinases (Röhm GmbH), and Pronase (Merck AG, Darmstadt).

*Reactive Site.* Trypsin inhibition activity of HUSI-I fraction D was not diminished by treatment with excess maleic anhydride and 2,3-butanedione reagent which are used for the identification of lysine or arginine residues in biologically active regions of proteins.[30] This indicates that neither a lysine nor an arginine residue is present in the reactive site of this inhibitor.

*Kinetic Properties.* The following dissociation constants ($K_i$) for the respective complexes were calculated according to Green and Work.[31]

| | | |
|---|---|---|
| HUSI-Id: complex with | bovine α-chymotrysin | $5 \times 10^{-10}$ *M* |
| | bovine trypsin | $4 \times 10^{-9}$ *M* |
| HUSI-II: complex with | bovine trypsin | $1 \times 10^{-9}$ *M* |
| | human acrosin | $9 \times 10^{-10}$ *M* |

*Immunology.* Antibodies produced by immunization of rabbits with HUSI-I (step 4, fractions A–D) cross-react with the trypsin–chymotrypsin inhibitor obtained from human spermatozoa and with the acid-stable trypsin–chymotrypsin inhibitor from cervical mucus,[32,33] but not with HUSI-II fractions A–D.

*Possible Biological Function.*[32] We assume, that the human seminal plasma inhibitor HUSI-I belongs to a special class of trypsin–chymotrypsin inhibitors including the inhibitors from respiratory tract secretions[23,34] and the inhibitor from cervical mucus.[33] These inhibitors form strong complexes with neutral proteinases from leukocytes and may protect mucous membranes against the hydrolytic action of these enzymes.

On the basis of the inhibition properties it may be concluded that HUSI-II, the trypsin–acrosin inhibitor in human seminal plasma, is the natural antagonist of the sperm acrosin.

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