Pepsin Inhibitors from Ascaris lumbricoides

ISOLATION, PURIFICATION, AND SOME PROPERTIES*

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SUMMARY

Extracts of the body walls of the adult Ascaris lumbricoides var. suis inhibit pepsin between pH 1 and 6. Four inhibitors of pepsin were isolated and purified as follows. The crude extract of Ascaris was incubated at 37° and pH 2.0 for 75 min. The pepsin-inhibiting activity was obtained in a fraction precipitated at 0.65 saturation with ammonium sulfate at pH 5.35, and sequentially chromatographed on Bio-Gel P-30 at pH 2.1 and Cellex SE at pH 4.7 and resolved into four inhibitors on DEAE-Sephadex at pH 8.8. The degree of purity of each inhibitor was demonstrated by disc gel electrophoresis. The over-all yield of pepsin inhibitors was 44% of the initial extract. Inhibitor I was 7%; Inhibitor II, 10%; Inhibitor III, 20%; and Inhibitor IV, 7%. The over-all purification of Inhibitors I → III was 9,600-fold and that of Inhibitor IV, 3,300-fold. The molecular weights of these inhibitors were calculated from their amino acid composition and were in agreement with the values obtained from both 5% and 10% polyacrylamide gels in sodium dodecyl sulfate. The values obtained from the amino acid composition were: 17,515 (= 160 amino acid residues), 15,584 (= 142 amino acid residues), 16,124 (= 147 amino acid residues), and 31,719 (= 290 amino acid residues) for Inhibitors I, II, III, and IV, respectively. The NH₂-terminal amino acid residue of each of these proteins was histidine. The ability of each of these inhibitors to inactivate pepsin was lost by its treatment with either trypsin or chymotrypsin. Each inhibitor inhibited porcine, bovine, and human pepsins, and porcine gastricsin, but not human gastricsin.

The stoichiometric interaction of a serine proteinase with a protein other than a γ-globulin to form a unique complex which behaves as a new species of protein and is devoid of proteinase activity is a well known phenomenon. The ubiquity of these serine proteinase inhibitors is indicated in a few selected references (2-5). These proteinase-protein complexes, which may be unstable, have apparent dissociation constants less than 10⁻⁸ M; some of these complexes can regulate the level of the active form of an enzyme. An example of this function is the presence of the secretory pancreatic trypsin inhibitor in the zymogen granules of the pancreas which minimizes the premature activation of trypsinogen and then the activation of the other zymogens in the granules by trypsin. In contrast to these widely studied systems, there are no examples of the inhibition of pepsin (which is not a serine proteinase) by well characterized non γ-globulin proteins, although one of the activation products obtained in the conversion of pepsinogen to pepsin inactivates the milk-clotting activity of pepsin at pH 5.0. This polypeptide, obtained in the activation of pepsinogen, is digested and ineffective at pH 2.0 where pepsin functions in the process of digestion (6).

Werde et al. (7) observed the inhibition of pepsin by a trypsin-inhibiting factor obtained by ammonium sulfate fractionation of a potato extract; Hilliard and West (8) and Carsten and Pierce (9) observed the inhibition of pepsin by extracts of bovine pituitary. In none of these three reports was the pepsin-inhibiting fraction purified and characterized. Recently some organic compounds have been obtained from among the metabolites of certain strains of actinomycetes which inhibit pepsin (10, 11). These pepstatin-inhibiting metabolites have molecular weights of 685 and 643. The metabolite from Streptomyces argenticolan var. toyokoenensis is called pepetatin and contains six organic units connected through five secondary amide bonds. Its structure is isovaleryl-L-valyl-L-valyl-L-4-amino-3-hydroxy-6-methylheptanoic acid. The metabolite of Streptomyces EF-44-201 is called S-PI and it also contains twice as much valine as alanine in “addition to other fatty acids.”

This communication reports that protein inhibitors of pepsin, able to inactivate pepsin at pH 2.0, have now been obtained from Ascaris lumbricoides. These substances were first recognized in 1910 by Mendel and Blood (12) who showed that extracts of Ascaris inhibited pepsin as well as trypsin, but not the plant proteinase, papain. Collier (13) obtained a trypsin-inhibiting preparation from Ascaris which also inhibited pepsin. When

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the trypsin-inhibiting fraction was purified further by treatment with hot 2.5% trichloroacetic acid and fractionated with ammonium sulfate, the pepsin-inhibiting activity was lost. Rola and Pudlec (14) also prepared an extract of *Ascaris* able to inhibit pepsin but following treatment with trichloroacetic acid they, too, lost the pepsin-inhibiting activity.

We are now reporting the isolation of four pepsin inhibitors. Each of these inhibitors can form a stable stoichiometric complex with pepsin capable of being isolated (15). The purity of these pepsin inhibitors will be demonstrated and their molecular weight, amino acid composition, stability in the presence of other proteolytic enzymes, and their interaction with acid proteinases from stomachs of different species reported.

### EXPERIMENTAL PROCEDURE

#### Materials

*Ascaris lumbricoides* var suis were collected at a slaughterhouse and were kept live in the salt medium of Baldwin and Moyle (16) at 37–40°C. The body walls of the adult worms were prepared as described by Peanasky and Laeckowski (17). Porcine pepsin (EC 3.4.2.1) twice crystallized with an activity of 2500 units per mg, bovine carboxypeptidase A (EC 3.4.1.2), chymotrypsin (EC 3.4.4.6), chymotrypsinogen A, and trypsin (EC 3.4.4.1) were purchased from Worthington. Pepsinogen (technical) from hog stomach mucosa was obtained from Nutritional Biochemicals, Cleveland, Ohio. All other chemicals were reagent grade. Double-distilled water was used throughout.

#### Methods

**Euglycemic Assay**—The activity of the inhibitor(s) was measured during purification studies by incubating 1 to 5 μg of inhibitor with 6 μg of pepsin in 10 mM acetate adjusted to pH 2.0 in a final volume of 1.0 ml at 37°C for 3 min. Residual pepsin activity was determined by adding 1.0 ml of 2.5% hemoglobin solution to each tube at 37°C and stopping the reaction after 5 min by adding 2.0 ml of cold 5% trichloroacetic acid solution. After standing in an ice bath for 15 to 30 min, the tubes were centrifuged for 40 min at 20,000 × g in a Sorvall angle centrifuge. The absorbance of the clear supernatant was determined at 280 nm in a Beckman model DU spectrophotometer and was proportional to the digestion of hemoglobin by pepsin. One unit of inhibitor was defined as that amount of inhibitor which inactivates 1.0 μg of pepsin under the conditions of the assay. Specific activity was defined as units of inhibitor per 1.0 unit of absorbance of the protein as measured through a 1-cm light path at 280 nm.

Carbohydrates were determined as hexoses and pentoses by the anthrone method (23). Disc gel electrophoresis was performed by the method of Ornstein and Davis (24).

Molecular weights were determined in 5% and 10% polyacrylamide gels in sodium dodeyl sulfate following the method of Dunker and Schachter (25).

NH₂-terminal analysis was done by Edman’s method as described by Schroeder (26).

Performic acid oxidation of protein samples was done by the procedure of Hirs (27).

Sulphydryl groups were determined by Ellman’s reagent in the presence of either sodium dodeyl sulfate, urea, or both (28).

Spectral analysis of essentially salt-free solutions of pepsin inhibitors was done at pH 2.0 (in one case at pH 7.0) with a Cary model 11 recording spectrophotometer.

**Amino Acid Analysis**—The amino acid composition of the inhibitors was determined on a Beckman-Spinco model 116 C amino acid analyzer following the method of Moore and Stein (29). The buffers were made in double-distilled water passed through a resin column and were washed with 3 void volumes (6 liters per void volume) before the application of the protein sample. The volume of the protein solution applied to the column was 4% of the volume of the sample.
gel in the column (250 ml) and the concentration of the protein charged was 8 to 12 A per ml. The column was operated by gravity flow at a rate of 180 ml per hour.

**Chromatography on Cellex SE—Cellex SE was prepared in 20 mM Tris-HCl. A column (2.4 X 45 cm) was equilibrated and washed with this solution before it was used. The protein solution was adjusted to 0.5 A per ml in 20 mM Tris-HCl, pH 3.65, before it was charged onto the column.** (When the solution was too dilute, it was concentrated on a UM-05 membrane under 40 p.s.i.)

**Chromatography on DEAE-Sephadex A-25—Dry DEAE-Sephadex A-25 was soaked in 100 mM Tris-HCl buffer, pH 8.8, for 48 hours at room temperature. A column (0.9 X 60 cm) was packed and equilibrated with the same buffer before it was used. All columns were operated in a cold room at 7° unless otherwise indicated.

**RESULTS AND DISCUSSION**

*Isolation of Pepsin Inhibitors*

The possibility that the pepsin inhibitor was uniquely located in the body walls of *Ascaris* was considered, but no difference was found in the distribution of pepsin-inhibiting activity when the worm was subdivided into three equal parts (heads, middles, and tails). Therefore, entire *Ascaris* from which the urogenital tract had been dissected were used in the preparations.

**Step 1. Ammonium Sulfate Fractionation**—Fig. 1 summarizes the steps employed to obtain the ammonium sulfate fraction of the pepsin inhibitor. Special precautions must be taken in separating the residue and the last 20 ml of the supernatant solution from the centrifugate at 50,000 X g. Careless decantation results in the addition of unwanted water-soluble materials which adversely affect the succeeding chromatography steps.

Attempts to extract or precipitate the inhibitors with trichloroacetic acid solution (as was described for trypsin inhibitors by Collier (13) and by Rola and Pudles (14)) failed. Instead, it was discovered that trichloroacetic acid inactivates both pepsin and the inhibitors.

The inhibitors are not precipitated in 70% MgSO₄ or in 70% ethanol, and are poorly precipitated in 90% acetone.

**Step 2. Chromatography on Bio-Gel P-30**—The ammonium sulfate fraction from Step 1 was applied to a Bio-Gel P-30 column, as summarized in Fig. 2.

The inhibitors were found on the descending side of the major protein peak. Carbohydrate was separated from the major protein peak and this facilitated the purification of the inhibitors in the next step. After the P-30 column was used several times, the separation of the proteins became poor. This was due to the irreversible absorption of the protein onto the column, i.e. about 70% of the first sample of protein charged into a fresh column was retained on the gel. Attempts to regenerate the column by boiling the gel at pH 8.0 and at high salt concentrations and at high and low pH were unsuccessful. Therefore, pepsin-inhibiting fractions were collected from this column until the specific activity of the combined fractions reached a limiting specific activity of 40 to 50. These fractions were concentrated on the UM-05 membrane and rechromatographed on a previously unused P-30 column (4.5 X 50 cm) under the same conditions for elution as the first column. This recycling step was effective, and a final recovery of better than 70% of the pepsin-inhibiting activity with a specific activity of the combined fractions of >120 was obtained.

**Step 3. Chromatography on Cellex-SE**—Fractions of pepsin inhibitors from Step 2 were applied to a column of Cellex SE as summarized in Fig. 3. Seventy per cent of the pepsin-inhibiting activity was eluted in a small peak before 40% of the protein applied was eluted.

**Step 4. Chromatography on DEAE-Sephadex**—Pepsin-inhibiting fractions from the Cellex column were concentrated, adjusted to pH 8.8, and charged onto a DEAE-Sephadex column. Fig. 4 shows the elution pattern. Each of the four peaks contained pepsin inhibitors.

Peaks I to III have the same apparent specific activity (3020 to 3400), but Peak IV appears to have half of the specific activity (1680) of the other peaks. The yield and specific activities of the fractions obtained in the purification are shown in Table I.

Polyacrylamide gel electrophoresis was performed on each pepsin-inhibiting fraction. Peaks I and IV appeared as single components and were easily separated by electrophoresis. Peaks II and III migrated as one component even in 5 and 10% gels in sodium dodecyl sulfate. When these four pepsin inhibitor peaks were mixed and electrophoresed, only three bands were obtained (Fig. 5).

**Molecular Weights**

Fig. 6 shows the relative mobility of pepsin Inhibitors I, II, and III in 5 and 10% polyacrylamide gel in sodium dodecyl sulfate. (Inhibitor IV was unavailable at this time and was not run.) The corresponding molecular weight was calculated as
### Table I

| Step of isolation          | Units of inhibitor | Specific activity | Recovery |
|----------------------------|-------------------|------------------|----------|
| 50,000 × g supernatant     | 126,000           | 0.5              | (100)    |
| Step 1 (ammonium sulfate fraction) | 100,800          | 6                | 80       |
| Step 2 (Bio-Gel P-30)      | 81,000            | 120              | 65       |
| Step 3 (Cellex SE)         | 64,000            | 1000             | 50       |
| Step 4 (DEAE–Sephadex)     |                   |                  |          |
| Peak I                     | 9,000             | 3020             | 7        |
| Peak II                    | 13,000            | 3400             | 10       |
| Peak III                   | 25,000            | 3400             | 20       |
| Peak IV                    | 10,000            | 180              | 7        |

Fig. 3. Chromatography on Cellex SE of *Ascaris* pepsin-inhibiting fraction from Bio-Gel P-30 column. Seventy A at 250 nm of total protein (= 10,000 units of inhibitors) were applied to a column (2.4 × 45 cm) which was equilibrated and eluted with 20 mM Tris-HCl-0.1 mM acetic acid solution at pH 3.65. At Fraction 30 elution was continued with 20 mM Tris-HCl-0.1 mM acetic acid, pH 4.6. At Fraction 125 a five-chamber gradient was used in which each of the first three chambers contained 300 ml of 20 mM Tris-HCl-0.1 mM acetic acid, pH 4.6, and the last two chambers contained 10 mM Tris-HCl-10 mM acetic acid, pH 5.0. Twelve-milliliter fractions were collected at a flow rate of 17 ml per hour. Ordinate is per cent of charge of protein (—) or per cent of charge of pepsin inhibitor (O—O).  

Fig. 4. Chromatography of *Ascaris* pepsin inhibitors on DEAE-Sephadex (A-25) after chromatography on Cellex SE. Inhibitor, 28.7 A (= 64,000 units of inhibitors), in 100 ml of 100 mM Tris-HCl buffer, pH 8.8, was applied to the column (0.9 × 60 cm) which was equilibrated with 100 mM Tris-HCl buffer, pH 8.8, at a rate of 14 ml per hour. After the protein solution passed into the gel, elution was continued with the same buffer. As soon as the absorbance at 280 nm dropped to the baseline (Position A), the elution was continued with a five-chamber gradient system in which the chambers contained 300 ml of 100, 200, 300, 400, and 500 mM Tris-HCl buffer, pH 8.8, respectively. At Position B, elution was continued with 500 mM Tris-HCl-10 mM KCl, pH 8.8. Ordinate is per cent of charge of protein (—) or per cent of charge of units of pepsin inhibitors (O—O).  

Fig. 5. Polyacrylamide gel electrophoresis of *Ascaris* pepsin inhibitors after chromatography on DEAE-Sephadex. Approximately 50 µg of inhibitor were applied to 7.5% cross-linked gels at pH 9.05. Electrophoresis was performed for 60 min in Tris-glycine buffer, pH 8.9 and 25°C. The gels were stained in 1% aniline blue-black and destained in 7% acetic acid. Protein bands migrated towards the anode. A is Peak I, B is Peaks II and III, C is Peak IV, and D is a mixture of Peaks I to IV.  

15,800 to 16,800 on the 5% gel (Fig. 6A) and 14,200 on the 10% gel (Fig. 6B). The accuracy of the determination of the molecular weight of a protein by this method was found to vary from ±1.5 to ±9.7% of the actual value (25). The proteins which
were chosen in this study were in this category. Therefore, we felt that an assignment of 15,500 (average of 14,200 and 16,800) for the molecular weight of the pepsin inhibitors (I, II, or III) could be within the mean of the experimental error.

### Amino Acid Analysis

Table II shows the amino acid composition of each inhibitor. Half-cystine residues were assigned from the analysis for cysteic acid following oxidation with performic acid. No free sulphydryl groups were found when the inhibitors were tested with Ellman's reagent even in the presence of urea and sodium dodecyl sulfate for 1 day. From this it was concluded that all of the half-cystine residues existed in the intact molecule of each inhibitor as disulfide bonds. No hexosamines were detected when the long column run of the amino acid analyzer was extended.

The minimal molecular weights calculated from amino acid compositions of these inhibitors were 17,515 (= 160 amino acid residues), 16,124 (= 147 residues), and 31,719 (= 290 residues) for Inhibitors I, II, III, and IV, respectively. Those values for Inhibitors I, II, and III were in agreement with the values obtained by the polyacrylamide gel method. The finding that Inhibitor IV had about twice the molecular weight of any of the other inhibitors was supported by the finding that Inhibitor IV had half of the specific activity of the other inhibitors. The inhibitor exhibited one band on polyacrylamide gel electrophoresis. The possibility that Inhibitor IV exists as a dimer with one active center is not likely because a number of the amino acid residues in this inhibitor are not double their number in any of the other inhibitors or any combination of two of the inhibitors. For example, Inhibitor IV has 31 alanine residues, while Inhibitors II and III each have 10 and Inhibitor I has 12 residues. Discrepancies can be found in comparing half-cystine, glycine, isoleucine, methionine, phenylalanine, and valine. The possibility that Inhibitor IV is a combination dimer of one of these three inhibitors and a fourth one, or a dimer of a fourth inhibitor, is not ruled out.

Since Inhibitors II and III could be separated (at least partially) by DEAE-Sephadex and not by disc gel electrophoresis, it was speculated that these two inhibitors might differ in their amino acid composition by a minimum number of amino acid residues. The amino acid analysis of both inhibitors revealed that Inhibitor III might have 1 more residue of aspartic acid (or asparagine), proline, and valine, and 2 more residues of glutamic acid (or glutamine) than Inhibitor II. It is likely that the acidic residues were asparagine and glutamine because a separation on disc gel electrophoresis was not achieved. This led to the conclusion that the separations of Inhibitors II and III on DEAE-Sephadex were due to stronger hydrophobic interaction between the DEAE-Sephadex matrix and Inhibitor III, which is richer in the hydrophobic amino acid residues than Inhibitor II.

### Spectral Analysis

The spectra of solutions of the four inhibitors (at pH 2.0) are shown in Fig. 7. The fine structure of phenylalanine, the broad maximum of tryptophan (276 to 283 nm), and the shoulder at 260 nm in every spectrum indicate the presence of these two aromatic amino acids in each of the four inhibitors.

The spectrum of a mixture of cystine, phenylalanine, tyrosine, and tryptophan in the molar proportions 3:10:1:1 at pH 2.0 is shown in Fig. 7D for comparison with the spectra of the other four inhibitors. The maximum at 263 nm is missing in the spectrum of each inhibitor. There are no differences in the spectra of these four Ascaris pepsin inhibitors. If Inhibitor IV had 4 tyrosine residues and 1 tryptophan, we might observe a sharper maximum in the spectrum at 275 nm. This argument supports the assignment of 2 tryptophan residues to the molecule. Wettlaufer (39) pointed out that the validity of the assignment of the aromatic amino acids in a protein, particularly tryptophan and tyrosine, may be checked by the ratio of the observed molar absorption, $e_{365}$, to the calculated molar absorption, $e_{365}$, of the protein, which theoretically should be 1.00. The ratio of $e_{365}$ to $e_{365}$ was computed from the observed molar absorption of the inhibitor at 280 nm and from its calculated molar absorption ($e_{365}$ = 5550, $e_{365}$ = 1340, and $e_{365}$ = 150, all at pH 2). Table III shows the $e_{365}$/ $e_{365}$ ratios of each Ascaris pepsin inhibitor and of a number of proteins listed for comparison. The ratio of 1.00 supports the assignment of 2 tryptophan residues per molecule of Inhibitor IV. The ratio 1.16 for Inhibitors II and III is within the normal value when compared to carbonic anhydrase (1.13) or to ribonuclease (1.13), and less than that obtained for atrazine (3-ketosteroid isomerase (1.37). The value 1.31 for Inhibitor I is still acceptable and is supported by the finding that this inhibitor exhibits a specific activity of 3020 compared to 3400 for either II or III.

### NH$_2$-terminal Analysis

The reaction of phenylisothiocyanate with each Ascaris pepsin inhibitor produced PTH-histidine. This indicated that the NH$_2$-terminal amino acid in each inhibitor was histidine.

### Stability of Pepsin Inhibitors

One of the characteristics which protease inhibitors exhibit is their resistance to the proteolytic action of other enzymes. Kassell and Laskowski (33, 34) found that the basic trypsin inhibitor (bovine) was not inactivated by pepsin or by chymotrypsin. Chymotrypsin inhibitors from Ascaris also were not inactivated by either trypsin or pepsin (89). We examined this phenomenon with pepsin inhibitors from Ascaris. Fig. 8 shows the inactivation of pepsin Inhibitor III by chymotrypsin and by trypsin (this also was true for Inhibitors I, II, and IV). When the enzyme and the inhibitor were mixed in 1:1 molar ratio at pH 7.5
against species specificity of the pepsin inhibitors was therefore tested related to the survival of the adult Ascaris in its proper host. The trypsin and they suggested that this observation could be re-

tors isolated from pork Ascaris were unable to inhibit human trypsin. The presence of chymotrypsin and $37^\circ$ for 1 hour the inhibitor was almost 100% inactivated in the presence of chymotrypsin and nearly 70% inactivated by trypsin.

Interaction of Ascaris Pepsin Inhibitors with Bovine and Human Gastric Enzymes

Pennasky and Abu-Erreish (35) observed that trypsin inhibitors isolated from pork Ascaris were unable to inhibit human trypsin and they suggested that this observation could be related to the survival of the adult Ascaris in its proper host. The species specificity of the pepsin inhibitors was therefore tested against bovine and human gastric enzymes.

Bovine pepsin was inhibited by each of the four Ascaris inhibitors, just like porcine pepsin. The inhibition of human and porcine pepsins by each of these Ascaris pepsin inhibitors is compared in Table IV. The inhibitors interact with porcine and with human pepsins in a stoichiometric (1:1 molar) ratio. A comparison of the inhibition of human and porcine gastricsin by these Ascaris pepsin inhibitors shows that porcine gastricsin is inhibited stoichiometrically while human gastricsin is not (Table IV). This is the second example of a species specificity which has been observed at the molecular level and which is in agreement with the host specificity of this parasite. Experiments are currently underway to relate these observations to an explanation of parasitism on a molecular basis.

Some other studies of the specificity of these pepsin inhibitors

| Amino Acid | Inhibitor I | Inhibitor II | Inhibitor III | Inhibitor IV |
|------------|-------------|--------------|---------------|--------------|
|            | 24 Hr       | 64 Hr        | 24 Hr         | 64 Hr        | 24 Hr        | 64 Hr        | 24 Hr        | 64 Hr        |
| Lysine     | 9.7         | 9.9          | 10            | 8.9          | 8.6          | 9             | 8.0          | 9.0          | 9             | 22.0         | 21.4         | 22            |
| Histidine  | 3.1         | 3.2          | 3             | 2.7          | 2.7          | 3             | 2.5          | 3.0          | 3             | 5.1          | 5.1          | 5             |
| Arginine   | 3.1         | 3.2          | 3             | 3.0          | 2.6          | 3             | 2.6          | 2.8          | 3             | 4.8          | 4.7          | 6             |
| Aspartic Acid | 14.5       | 14.9         | 15            | 13.9         | 13.3         | 14            | 14.7         | 14.4         | 15            | 29.8         | 29.2         | 30            |
| Threonine  | 8.8         | 8.5          | 9 $^a$        | 7.6          | 6.6          | 8 $^a$        | 8.1          | 7.5          | 8 $^a$        | 15.2         | 14.5         | 16 $^a$       |
| Serine     | 6.6         | 5.5          | 7 $^a$        | 6.0          | 4.5          | 7 $^a$        | 5.4          | 4.9          | 6 $^a$        | 10.9         | 9.2          | 12 $^a$       |
| Glutamic Acid | 20.6       | 21.9         | 22 $^b$       | 17.3         | 16.4         | 17            | 17.1         | 19.2         | 19 $^b$       | 41.2         | 42.6         | 42            |
| Proline    | 15.8        | 18.4         | 18 $^b$       | 14.3         | 16.8         | 17 $^b$       | 13.8         | 18.5         | 18 $^b$       | 25.2         | 26.8         | 27 $^b$       |
| Glycine    | 10.3        | 10.9         | 11            | 9.3          | 8.6          | 9             | 10.0         | 9.7          | 10            | 17.0         | 17.3         | 17            |
| Alanine    | 12.0        | 11.9         | 12            | 9.9          | 9.6          | 10            | 9.9          | 9.8          | 10            | 31.2         | 31.1         | 31            |
| Half-Cystine | 5.4         | --           | 6 $^c$        | 4.7          | --           | 6 $^c$        | 4.9          | --           | 6 $^c$        | --           | 6.6          | --           |
| Valine     | 11.4        | 12.6         | 13 $^b$       | 11.0         | 10.2         | 11            | 11.6         | 11.4         | 12            | 17.0         | 18.6         | 19 $^b$       |
| Methionine | 4.7         | --           | 5 $^d$        | 3.8          | --           | 4 $^d$        | 3.9          | --           | 4 $^d$        | 5.7          | --           | 6 $^d$       |
| Isoleucine | 4.0         | 4.0          | 4             | 3.7          | 3.2          | 4             | 3.2          | 3.5          | 4 $^d$        | 10.0         | 10.2         | 10            |
| Leucine    | 7.3         | 7.8          | 8             | 6.7          | 6.5          | 7             | 6.8          | 6.8          | 7             | 15.2         | 15.8         | 16            |
| Tyrosine   | 1.0         | 1.2          | 1             | 1.0          | 1.0          | 1             | 0.9          | 1.1          | 1              | 2.2          | 3.5          | 4 $^d$       |
| Phenylalanine | 11.8       | 12.3         | 12            | 10.9         | 10.0         | 11            | 10.5         | 11.4         | 11            | 18.1         | 18.1         | 18            |
| Tryptophan | --          | 0.8          | 1 $^b$        | --           | 0.7          | 1 $^b$        | --           | 1.0          | 1 $^b$        | --           | 1.1          | 2 $^c$       |
| Total Residues | 160        | 142          | 147          | 160          | 142          | 147          | 160          | 142          | 147          |
| Ammonia    | (19)        | (17)         | (17)          | (19)         | (17)         | (17)          | (19)         | (17)         | (17)          |
| Molecular Weight | 17,515   | 15,584     | 16,124       | 31,719       | 17,515 | 15,584     | 16,124       | 31,719       |

$^a$ Obtained by extrapolation to zero time.
$^b$ 64-hour hydrolysate only.
$^c$ As cysteic acid from a 24-hour hydrolysate following performic acid oxidation.
$^d$ As methionine sulfoxide from a 24-hour hydrolysate following performic acid oxidation.
Fig. 7. Ultraviolet absorption spectra of Ascaris pepsin inhibitors. Spectra were scanned on a Cary model 14 Spectrophotometer at a speed of 0.25 nm per s. All solutions were measured through a 1-cm light path and were in 10 mM HCl (pH 2.0) except C-a which was in 0.1 mM Tris-HCl, pH 7.0. A, inhibitor I; B, inhibitor II or III; C-b, inhibitor IV; C-a, inhibitor IV (pH 7.0); D, solution of $6.3 \times 10^{-4}$ M cystine, $21 \times 10^{-4}$ M phenylalanine, $2.1 \times 10^{-4}$ M tyrosine, and $2.1 \times 10^{-4}$ M tryptophan, all at pH 2.0.

FIG. 8. Inactivation of Ascaris pepsin inhibitors with intestinal proteinases. Pepsin inhibitor ($9 \times 10^{-4}$ amoles) and enzyme ($9 \times 10^{-3}$ amoles) were mixed in 200 mM Tris-HCl, pH 7.6, and incubated at 37°C. One hundred-microliter aliquots were removed at the indicated times and the pH was adjusted to 2.0. Pepsin inhibitor activity was determined against 6 pg of pepsin using the hemoglobin assay method. Pepsin inhibitor + chymotrypsin, $\Delta-\Delta$; pepsin inhibitor + trypsin, $\triangle-\triangle$.

TABLE IV
Comparison of inhibition of porcine and human pepsins by Ascaris pepsin inhibitors*  

| Enzyme         | Ascaris pepsin inhibitor | Inhibitor added | Enzyme, inhibited | Ratio enzyme inhibited to inhibitor added | Enzyme inhibitor | Inhibitor added | Enzyme, inhibited | Ratio enzyme inhibited to inhibitor added |
|----------------|--------------------------|-----------------|-------------------|------------------------------------------|-----------------|-----------------|-------------------|------------------------------------------|
|                |                          |                 |                   |                                          |                 |                 |                   |                                          |
| Porcine pepsin |                          |                 |                   |                                          |                 |                 |                   |                                          |
| I              | 0.80                     | 0.81            | 1.00              |                                          |                 |                 |                   |                                          |
| II             | 1.44                     | 1.40            | 0.97              |                                          |                 |                 |                   |                                          |
| III            | 1.03                     | 1.00            | 0.97              |                                          |                 |                 |                   |                                          |
| IV             | 1.60                     | 1.61            | 1.00              |                                          |                 |                 |                   |                                          |
| Human pepsin   |                          |                 |                   |                                          |                 |                 |                   |                                          |
| I              | 0.80                     | 0.80            | 1.00              |                                          |                 |                 |                   |                                          |
| II             | 1.44                     | 1.34            | 0.93              |                                          |                 |                 |                   |                                          |
| III            | 1.96                     | 1.73            | 0.88              |                                          |                 |                 |                   |                                          |
| IV             | 1.60                     | 1.37            | 0.86              |                                          |                 |                 |                   |                                          |
| Human gastricsin|                          |                 |                   |                                          |                 |                 |                   |                                          |
| I              | 0.80                     | 0.27            | 0.32              |                                          |                 |                 |                   |                                          |
| II + III       | 12.70                    | 0.40            | 0.03              |                                          |                 |                 |                   |                                          |
| IV             | 1.60                     | 0.07            | 0.04              |                                          |                 |                 |                   |                                          |
| Porcine gastricsin |                          |                 |                   |                                          |                 |                 |                   |                                          |
| I              | 1.73                     | 1.51            | 0.87              |                                          |                 |                 |                   |                                          |
| II + III       | 1.35                     | 1.46            | 1.08              |                                          |                 |                 |                   |                                          |
| IV             | 1.78                     | 1.48            | 0.83              |                                          |                 |                 |                   |                                          |

* Pepsins, gastricsins, and inhibitors were allowed to react at pH 2.0 and 37°C for 5 min. Free pepsin or gastricsin was estimated by the rate at which it hydrolyzed hemoglobin for 5 min.

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