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PEPSIN INHIBITOR FROM ROOTS OF *ANCHUSA STRIGOSA*

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Key Word Index—*Anchusa strigosa*; Boraginaceae; pepsin inhibition; acid protease inhibitor; protease inhibitor; gastric ulcer; stomach ulcer.

Abstract—A pepsin inhibitor of undetermined exact chemical composition was isolated from the aqueous extracts of the roots of *Anchusa strigosa*. The extract of 1 g dry roots inhibited 9380 ± 390 μg of pepsin. The main steps of the isolation procedure consisted of extraction in boiling water, precipitation at pH 3 and washing the precipitate with ethanol, column chromatography on sephadex G-75, and finally ultrafiltration on UM10 membrane. The specific activity at the last step was 44 units ml^{-1} solution which measured 1.0 at 287 nm (a one unit of the inhibitor is that amount which inhibited 1 μg of pepsin under the conditions of the assay). The overall purification was 22-fold. The inhibitor did not bind CM-cellulose, but it did bind DEAE-cellulose irreversibly. A pepsin-inhibitor complex was formed with a $K_I = 2 \times 10^{-8}$ M. The inhibitor inhibited peptic milk-clotting activity at pH 5.3. A solution of the inhibitor exhibited split-peak spectra with two maxima at 280 nm and 287 nm. The ε at 287 nm was estimated at 9.4×10^5 l mol^{-1} cm^{-1} based on a M_r value which was estimated at 62,500.

INTRODUCTION

A number of substances have been known to inhibit pepsin activity. Of these are the basic polypeptide [1], synthetic sulfated polysaccharide known as C-16 [2], substrate analogues [3, 4], microbial metabolites such as pepstatin from *Actinomycetes* [5] and S-PI from *Streptomyces* [6] and inhibitors from *Ascaris lumbricoides* [7]. The role of pepsin in ulcerogenesis is not well defined, and no single anti-ulcer drug could give complete healing in cases treated without recrudescence [8]. Several reports have dealt with the gastric protection or peptic ulcer treatment using plant extracts against experimental induced gastric ulcer [9, 10]. Aqueous extracts of *Anchusa strigosa* roots have shown gastric protection and healing of ethanol-induced stomach ulcer in experimental animals [unpublished results]. The mechanism of the root extract effect, whether protective or curative, on ethanol-induced gastric ulcer has been thought of as inhibition of pepsin activity by the extract. Therefore, studies on the inhibition of the peptic activity by the root extract of *Anchusa strigosa*, and the partial characterisation of the inhibitory component have been the subject of this study.

RESULTS AND DISCUSSION

Isolation of Anchusa strigosa *pepsin inhibitor (ASPI)*

The aqueous extract of the *A. strigosa* roots at 42° was essentially free of pepsin inhibition activity. The inhibitory fraction was found in the extract of boiling water. The level of the inhibitor activity in the extract was linearly proportionate to the weight of the dry root in the extract which indicated complete extraction of the inhibitor under the conditions of the extraction procedure. The light absorption spectra showed that the extract had a peak at 287nm with a shoulder at 280 nm and this was taken as a temporary characteristic reference for the inhibitor. Table 1 represents typical results of the light absorption at 287 nm of the extract, μg of pepsin inhibited (= units of inhibition)

Table 1. Pepsin inhibitor activity in the crude extract of the roots of *Anchusa strigosa*. Extract preparations were in triplicates

g dry roots/l	A^a based on 1.0 g/l	Units^b/A	Units/g
10	4.70 ± 0.20^c	2.10 ± 0.10	9640 ± 480
20	4.40 ± 0.20	2.20 ± 0.10	9770 ± 490
30	5.00 ± 0.20	1.90 ± 0.10	9290 ± 450
Average	4.60 ± 0.30	2.00 ± 0.10	9380 ± 390

^{a} Absorbance at 287 nm.

^{b} Unit of inhibitor activity is the amount of the inhibitor which inhibits 1 μg of pepsin under the conditions of the assay.

^{c} Data are in means ± s.d. Analysis of variance indicates no significant differences among the means of the values in the same column. $F = 2.02$; $F_{0.05(4,10)} = 3.48$.

by 1 ml extract that exhibited 1 A unit at 287 nm (specific activity) and the units of inhibition in 1 ml extract of a particular root weight per 1 liter extract. The specific activity, as units/ ml solution which measured 1 at 287 nm, and the units/g dry roots were 2.00 ± 0.20 and 9380 ± 390, respectively. The pH titration profile of the crude extract, which had pH 6.9, indicated the presence of a titratable group with a pK_a value of 4.3. At pH 3, a gelatine-like precipitated material was formed. The precipitate was separated by centrifugation, and was found to contain the pepsin inhibitory activity. The acid precipitate did not dissolve in acetone or in alcohol, however, brown and yellow colored substances were removed by alcohol. The precipitate readily dissolved in a solution at pH 6–7. Preparation of the acid precipitate from 42°–100° yielded the same quality and quantity of the acid precipitate obtained from the extraction once at 100°. Therefore, extraction at 42° was omitted. Chromatography of the acid-precipitate fraction on sephadex G-75 column resulted in three peaks (figure is not shown). The first peak contained the inhibition activity towards pepsin. The active fractions of the peak were combined, concentrated and desalted on UM10 membrane at 40 psi. The inhibitor did not bind CM-cellulose at pH 5; but it did bind DEAE-cellulose at pH 8. All attempts, including elution with high salt concentration, extreme low and high pH values, and the combinations of these failed, to dissociate it from the ion-exchanger. Table 2 summarises the main steps of the purification, the specific activity at each step, and the ratio of E_{287nm}/E_{260nm} for the inhibitor.

Characterisation of the pepsin inhibitor

The light absorption spectra profiles for the UM10 fraction at pH values of 5 and 11 were scanned (figure is not shown). The spectra retained the peak at both pH values. However, there was an increase in the amplitude of the signal at 287 nm without shifts in the wavelengths of the maxima. The UV absorption spectra of the inhibitor, pepsin, and the inhibitor-pepsin complex are presented in Fig. 1. The spectra indicate the presence of tyrosine-like and tryptophan-like and the absence of phenylalanine-like residues in the inhibitor. From the dry weight of a sample that inhibited a known quantity of pepsin, the relationship, $P + I \leftrightarrow P - I$, and the titration curve (Fig. 2) the apparent dissociation constant of the P – I complex, K_I, was calculated as 2×10^{-8} M for a M_r of 62,500 [11]. The ε at 287 nm was estimated as 9.4×10^5 l mol^{-1} cm^{-1}. The inhibitor also inhibited milk-clotting activity of pepsin at pH 5.3, however, the milk-clotting time increased when the inhibitor and the enzyme were allowed to react first at pH 2 before the addition of the buffered milk (data not shown). This suggests that the pepsin-inhibitor binding is pH-dependent (within the range of pH for pepsin activity) and the binding is reversible. The above results are similar to a certain extent to those obtained from the pepsin inhibitor from *Ascaris lumbricoides*, $K_I = 2 \times 10^{-9}$ M [12]. Trypsin, chymotrypsin, and elastase, which are alkaline and serine proteases, and CPase A were not inhibited by the crude extract or its subsequent fractions. This indicates that the inhibitor is an acid, not alkaline, protease inhibitor. The assay for protease activity in the crude extract and its subsequent fractions showed its absence. This was not surprising because the extract was prepared by boiling the aque-

Table 2. Summary of the steps of purification of *Anchusa strigosa* pepsin inhibitor. Three extracts of roots were used (10 g/l, 15 g/l, and 20 g/l, Table 1). The data represent the results of the three separate runs

Fraction	Specific activity units[a]/ml (A^b = 1.0)	Ratio of A at 287 nm/A at 260 nm
Crude 42°–100°	8.30 ± 0.40	1.80 ± 0.10
Acid precipitate	25.30 ± 1.30	3.10 ± 0.10
Sephadex G-75 UM10[c]	44 ± 2.20[c]	5.00 ± 0.10

[a] A unit of activity is the amount of the inhibitor which inhibits 1.0 μg of pepsin under the conditions of the assay.

[b] The absorbance, A, was measured at 287 nm.

[c] Purified 22-fold relative to the original crude extract which has specific activity of 2.00 (Table 1). Data are given in means ± s.d.

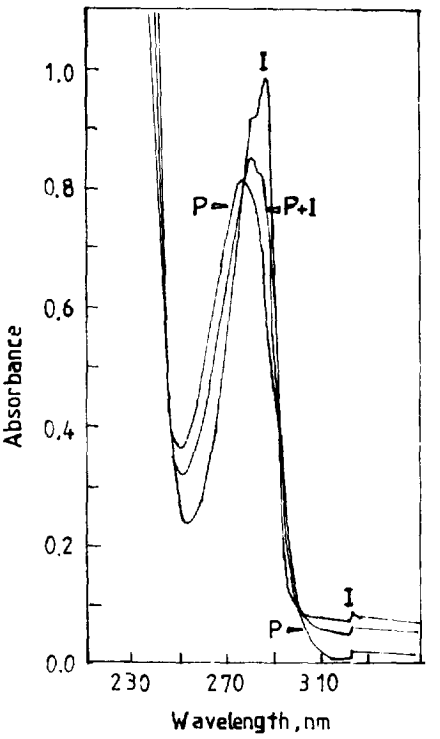

Fig. 1. Ultraviolet absorption spectra of *Anchusa strigosa* pepsin inhibitor-pepsin complex at pH 5.3: P = pepsin. I = inhibitor.

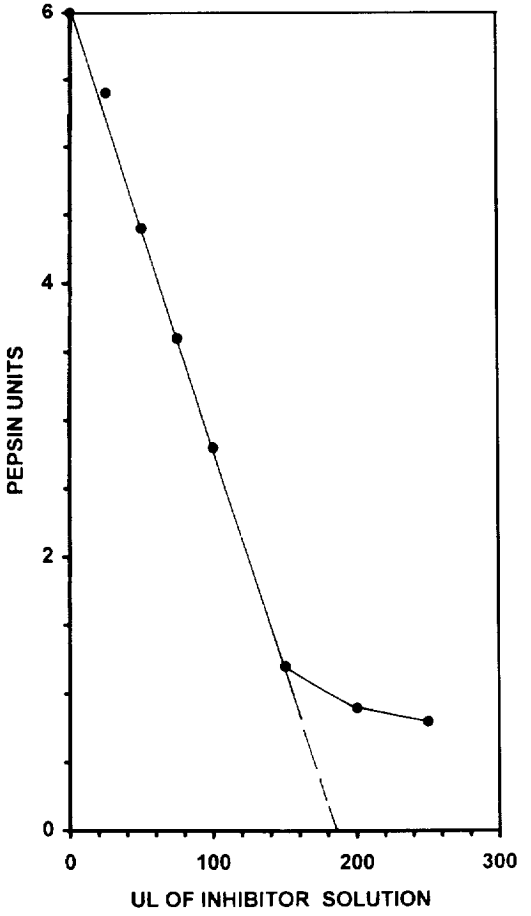

Fig. 2. Titration of pepsin with *Anchusa strigosa* pepsin inhibitor. Pepsin (6.0 μg) was titrated with known concentration of dilute inhibitor solution. Residual activity of pepsin was determined with haemoglobin assay as described in text.

ous suspension of the material. Ferric chloride, however, gave a whitish precipitate with the inhibitor. The assay for protein using biuret gave a greenish color. Folin-Lowry assay gave the actual blue color, but the low values obtained and the spectra did not indicate that the inhibitor is a polyphenolic compound. The anthrone test for hexoses and pentoses suggested that the inhibitor contains about 3.6% sugar. From the results provided above, the spectral properties and the chemical analysis, it could be concluded that the inhibitor is not similar to the known polyphenols [13]. It could be a polymer of aromatic residues and carbohydrates with uncommon modifications. Nevertheless, the inhibitor activity was highly stable whether the inhibitor was stored at room temperature or in the refrigerator. For example, after 7 months the crude extract had no loss of peptic inhibition activity. The acid precipitate and the Sephadex G-75-UM10 fractions lost < 14% of the activity at 4°. Finally an additional interesting feature of the inhibitor is that no any kind of microbial growth was observed in the crude extract and in its subsequent fractions for the same period under the same conditions mentioned above. These features may imply that it could be an excellent anti-ulcer-causative agents whether they are peptic or bacterial such as *Helicobacter pylori* [14]. With these features and its gelation property at the pH of the stomach cavity, treatment or prevention of gastric ulceration with the inhibitor is promising, and is currently this is under investigation.

EXPERIMENTAL

Plant material

The roots of *Anchusa strigosa* (Banks et Sol), Boraginaceae, were collected from the University of Jordan area. A voucher number 50 was deposited to the herbarium at the Department of Biological Sciences. The plant was identified by Al-Eisawi, a botanist at the department. The time of plant collection was during the summers of 1995–1996. The roots were air-dried and ground.

General

The following enzymes and reagents were used: bovine carboxypeptidase A (CPase, EC 3.4.17.1), chymotrypsin (EC 3.4.21.1), elastase (EC 3.4.21.37), trypsin (EC 3.4.4.4), porcine pepsin (EC 3.4.4.1), *N*-benzoyl-L-arginine ethyl ester (BAEE, substrate for trypsin), *N*-carboxyglycyl-L-phenylalanine (substrate for CPase), *p*-nitrophenyl acetate (substrate for chymotrypsin), succinyl-(alanine)$_3$-*p*-nitroanilide (substrate for elastase), CM-cellulose 32, DEAE-cellulose 23S, Sephadex G-75, and tris[hydroxymethyl]amino methane (Tris)-HCl. Haemoglobin was prepared from outdated human red cells from the University Hospital according to [15]

Preparation of roots extract. The aq. extract of the dry ground roots of the *Anchusa strigosa* was prepared as follows: (a) A root sample of known wt was first extracted with H_2O at 40° for overnight period. The soluble clear soln was separated from the residual material by centrifugation at 21,980 *g* for 20 min in an Universal Junior II KS angle refrigerated centrifuge. (b) The residue was made to the initial vol. by addition of H_2O and was subjected to extraction by boiling for 20 min. The clear extract was obtained by centrifugation as in the above procedure and stored at 4°.

Assay of pepsin inhibition. (a) Using haemoglobin as substrate at pH 2. Through the steps of isolation of the inhibitor, the inhibition of pepsin activity was measured as follows: A known vol. of the inhibitor soln was added to 6 μg of pepsin in 100 μl of 10 mM NaOAc buffer, pH 5.3. After 3 min, HCl soln was added to make the final vol. to 1 ml at pH 2. The mixture was incubated at 37° for 3 min. The residual pepsin activity was determined by adding 1 ml of 2.5%

haemoglobin soln (previously dialysed at pH 2 on PM30 membrane at under N_2 at 40 psi in an Amicon cell) at 37°. The reaction was carried out for 5 min, and was stopped by adding 2 ml of cold 5% TCA soln. After standing in an ice bath for ⩾ 30 min, the mixture was centrifuged at 30,000 *g* for 40 min to obtain a clear supernatant. Blanks and controls were prepared similarly by adding the pepsin, the inhibitor, or both after the addition of TCA soln. The *A* at 280 nm was measured in a Pye Unicam model SP8-400 U/V spectrophotometer and was proportional to the degree of digestion of haemoglobin by pepsin. 1 Unit of inhibitor activity was defined as the amount of inhibitor which inhibits 1 μg of pepsin under the conditions of the assay. The specific activity of the inhibitor was defined as the number of units of inhibitor per 1 ml of the inhibitor soln which gives 1 *A* at 287 nm in a cuvette of 1 cm light path [7]. The reproducibility error of the assay was set at ± ⩽5%.

(b) Using casein in milk as substrate at pH 5.3. Although pepsin hydrolyses proteins at pH 2, it still hydrolyses certain peptide bonds in casein at pH 5 to 6 and this causes milk-clotting in the presence of Ca^{2+}. The rate of milk-clotting is expressed in terms of milk-clotting time. The inhibition of milk-clotting activity of pepsin by the inhibitor was tested accordingly [16]. Blanks and controls were prepared similarly by adding the inhibitor, pepsin or both, to the reaction mixture.

Assay of inhibition of alkaline proteases. Inhibition of CPase A, chymotrypsin, elastase, and trypsin activities by the extract and its subsequent fractions were measured [17]. As an example, the activity of trypsin was measured at 25° in terms of the increase in the *A* at 253 nm due to the addition of 0.2 ml of the enzyme soln (0.35 mg ml^{-1}) to 2.8 ml of 50 mM Tris-HCl buffer, pH 8, containing 0.34 mg ml^{-1} BAEE substrate and 2.22 mg ml^{-1} $CaCl_2$. The assay for the inhibition was as above except that the enzyme was first incubated with the inhibitor fraction for 5 min before the addition of the buffered substrate. Appropriate blanks and controls were carried out in the presence and absence of the inhibitor and trypsin. Trypsin concentration in the soln was determined from the relationship of $\varepsilon^{1\%} = 15$ using 1 cm cuvette and 278 nm for the measurement. Similar procedures were followed for the inhibition assays for the other enzymes mentioned above.

Assay for protease activity in the roots extract. The inhibitor alone was incubated with the substrates (haemoglobin, milk, or the other substrates of the enzymes mentioned above) under the same conditions of the enzyme assay. The products were measured as indicated in the assays.

Protein determination. The biuret reaction [18] and the phenolic reagent [19] were used to measure protein.

Carbohydrate measurement. The anthrone reagent [20] was used to estimate carbohydrates as hexoses and pentoses.

Ion-exchange and gel filtration chromatography

Ion-exchangers, Sephadex-G, and the corresponding columns were prepared according to the brochures by the manufacturers.

Isolation of pepsin inhibitor from the roots extract

Step 1. *Precipitation of the inhibitor.* The fraction that contained pepsin inhibitor activity was precipitated in HCl soln at pH 3. The gelatine-like ppt. was separated from the soln by centrifugation, and washed with EtOH several times until the last EtOH addition was colorless. The ppt. was dissolved in a soln at pH 7 using NaOH to adjust the pH.

Step 2. *Chromatography on Sephadex G-75 and ion-exchangers.* The pepsin-inhibiting fraction of the acid-ppt. was treated as: (a) it was applied to Sephadex G-75 column. The column was equilibrated and eluted with 0.1 M KCl soln. Fractions were collected at constant pressure using a peristaltic pump. The active fractions from step 2-a were concentrated and desalted on UM10 Diaflo membrane. (b) from the last concentrated fraction a sample was passed into a DEAE 23S column at pH 8, and another sample was passed into a CM 32 column at pH 5.

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