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SPECIFICITY AND STABILITY OF THE CHYMOTRYPSIN INHIBITOR FROM WINGED BEAN SEED (*PSOPHOCARPUS TETRAGONOLOBUS* (L) Dc.)

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Summary

The specificity of the winged bean chymotrypsin inhibitor is restricted to the chymotrypsins (EC 3.4.21.1 and EC 3.4.21.2). Trypsins (EC 3.4.21.4), elastase (EC 3.4.21.11), subtilisins (EC 3.4.21.14), proteinase K (EC 3.4.21.14) and Pronase (EC 3.4.24.4) are not inhibited. The inhibitor reacts with two molecules of chymotrypsin to form a stable complex (M_r approx. 70000) which was isolated by gel filtration on Sephadex G-100. When mixed with substrate, the interaction of the inhibitor with α-chymotrypsin is characterized by substrate-induced dissociation of the complex. In contrast, the interaction with chymotrypsin B is quantitative with no substrate-induced dissociation. The inhibitor reacts with α-chymotrypsin to form a 1 : 2 molar complex at all ratios of [I]/[E]; however, the interaction with chymotrypsin B is characterized by the formation initially of a 1 : 1 molar complex at [I] > [E] followed by the formation of the 1 : 2 molar complex at [I] < 2[E]; an intermediate species of M_r approx. 48 000 was demonstrated by gel filtration on Sephadex G-100.

The inhibitor is stable over the pH range 2.0—11.5 and to heating up to 70°C at pH 4.1 and 8.0, and up to 90°C at pH 3.0. The inhibitor resists denaturation in 8.0 M urea at pH 8.0 and 4.0, and is stable in 0.12 M β-mercaptoethanol at pH 8.0; however, reduction in 8.0 M urea results in a loss of inhibitory activity. The inhibitor resists digestion with pepsin at pH 2.0, being only slowly degraded over a period of 7 days with an equimolar amount of pepsin.

Introduction

The presence of a protein inhibitor specific for bovine α-chymotrypsin in the seed of the winged bean (*Psophocarpus tetragonolobus* (L) Dc) was recently

documented and its isolation and properties described [1]. The winged bean chymotrypsin inhibitor is, to date, the only legume seed inhibitor which is capable of inhibiting bovine α-chymotrypsin but not bovine trypsin. The inhibitor has a molecular weight of 20 900 and contains four half-cystine residues/mol; it can be considered as another representative of the higher molecular weight (approx. 20 000), low half-cystine class of legume seed inhibitors such as the well known soybean trypsin inhibitor (Kunitz) [2]. The inhibitor was found to be double-headed, inhibiting two molecules of α-chymotrypsin simultaneously. However, the interaction of the inibitor with bovine α-chymotrypsin was not quantitative and substrate-induced dissociation of the inhibitor-enzyme complex was evident. The inhibitor-enzyme complex was isolated by gel filtration on Sephadex G-100 at pH 8.0 and was shown to have a molecular weight of about 70 000.

To fully characterize this novel inhibitor and to assess its role as an antinutritional factor of the winged bean, the interaction of the inhibitor with a series of serine proteinases was examined and the thermal and pH stability of the inhibitor was investigated. The results of this study are reported in this paper.

Materials and Methods

The winged bean chymotrypsin inhibitor was prepared as described earlier [1]. Bovine trypsin and α-chymotrypsin, and porcine pepsin were from Worthington. Bovine γ-, δ-, β-chymotrypsins, elastase (porcine), subtilisin (Carlsberg and BPN') and the synthetic substrates, α-*N*-benzoyl-L-tyrosine ethyl ester and α-*N*-benzoyl-L-arginine ethyl ester hydrochloride were from Sigma. Proteinase K and casein (Hammersten) were from Merck. Pronase was from Calbiochem-Behring Co. Bovine chymotrypsin B and porcine chymotrypsin A were prepared from pancreatic residues as described earlier [3]. Ovine α-chymotrypsin and chymotrypsin B were prepared from fresh ovine pancreas using the same preparative procedure [3]. All other chemicals used were analytical reagent grade and deionized water was used for all solutions.

Assay of enzymatic and inhibitory activities. The proteolytic activity of the various proteinases was measured by the casein digestion method of Kunitz [4] as modified by Kakade et al. [5]. The assay contained 10 mg casein in 1.95 ml 0.05 M Tris-HCl/0.01 M $CaCl_2$, pH 8.0, and 0.05 ml enzyme or enzyme/inhibitor mixture was added. After 10—20 min at 40°C, the reaction was stopped by adding 3.0 ml buffered trichloroacetic acid [5]. After standing at room temperature for 1 h the solution was filtered (Whatman No. 3) and the absorbance of the supernatant was measured at 280 nm.

The esterolytic activity of chymotrypsins, proteinase K and Pronase was assayed with α-*N*-benzoyl-L-tyrosine ethyl ester as substrate; the esterolytic activity of trypsins and also Pronase was assayed with α-*N*-benzoyl-L-arginine ethyl ester as substrate. The assays were performed as described earlier [1].

The inhibitory activity was estimated from the residual activity of a mixture of proteinase and inhibitor as described [1]. The concentration of the inhibitor was calculated from the absorbance at 280 nm and the molar extinction coefficient ($E^{1\%}_{1cm}$, 11.3). The concentrations of the chymotrypsin solutions

were calculated in a similar manner using the known molar extinction coefficients [6] and were corrected for inactive enzyme as described earlier [1].

Isolation and molecular weight estimations of the inhibitor-chymotrypsin complexes. The interaction of the inhibitor with the various chymotrypsins and the isolation of the complexes was carried out be gel filtration on Sephadex G-100. The inhibitor and enzyme were mixed at pH 8.00 (0.05 Tris-HCl/0.1 M NaCl) in a volume of 2.0 ml and after 10 min at room temperature applied to the column. The formation of the complexes and their isolation were carried out under conditions of (a) excess inhibitor and (b) excess enzyme as described in the Results. The column was calibrated with the following standard proteins; bovine serum albumin, ovalbumin, bovine chymotrypsinogen A, myoglobin and cytochrome *c*. A linear relationship was obtained when the logarithms of the molecular weights were plotted against the ratios of elution to void volume (V_e/V_o). The molecular weights of the complexes were estimated from the standard plot.

Electrophoretic method. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) was performed according to Weber and Osborn [7] in 7.5% polyacrylamide gels. Gels were stained with 0.25% Coomassie brilliant blue G-250 and destained by washing with 20% methanol/10% acetic acid.

Effect of pH on the stability of the inhibitor. The stability of the inhibitor was examined in the pH range of 2—12.2 at room temperature. The inhibitor (0.44 mg protein) was dissolved in 0.5 ml 0.1 M buffer (pH 2.0, HCl/KCl; pH 4.0, acetate; pH 6.0, citrate; pH 8.0, Tris-HCl; pH values 10.0, 11.5 and 12.0, ammonium bicarbonate; pH 12.2, 0.2 M NaOH) and the final pH of the solution measured. After 16 h, aliquots (50 μl) were removed and the pH adjusted to 8.0 for assay of inhibitory activity of chymotrypsin. At pH 11.5, 11.9 and 12.2 the inactivation of the inhibitor was followed as a function of time and the aliquots were removed at various times and assayed for inhibitory activity.

Effect of temperature on the stability of the inhibitor. The inhibitor (78.4 μg protein) in 0.1 ml buffer (0.1 M acetate, pH 4.0; 0.1 M Tris-HCl, pH 8.0; 0.001 M HCl, pH 3.0) was incubated in a sealed tube at various temperatures for 10 min then cooled in ice and 50-μl aliquots were assayed for inhibitory activity.

Effect of urea and β-mercaptoethanol on the stability of the inhibitor. The inhibitor (0.12 mg protein) in 0.02 ml H_2O was mixed with 0.2 ml buffer at pH 4.0 (0.1 M acetate) and at pH 8.0 (0.1 M Tris-HCl), containing urea (8 M, final concentration), and then treated as required (heating, time incubation, addition of β-mercaptoethanol). The effect of β-mercaptoethanol in the absence of 8 M urea was determined in the same manner. Aliquots (50 μl) of the treated inhibitor were removed and assayed for inhibitory activity. Suitable controls were run in the absence of inhibitor to assess the effect of urea and β-mercaptoethanol on the activity of bovine α-chymotrypsin in the assay.

Treatment of the inhibitor with pepsin. The inhibitor (0.25 mg) was treated with porcine pepsin (0.12 and 0.24 mg) at pH 2.0 (in 0.6 ml 0.02 M HCl/0.5 M KCl) at 37°C. At various times, 50-μl aliquots were withdrawn, pH adjusted to 8.0, and assayed as described above. At day 7 a sample was treated with an equal aliquot of 2% SDS/2% β-mercaptoethanol and examined by SDS-polyacrylamide gel electrophoresis.

Results and Discussion

The winged bean chymotrypsin inhibitor was found to inhibit the proteolytic and esterolytic activites only of the chymotrypsins including bovine and ovine chymotrypsins (α and B) and porcine chymotrypsin A. No inhibition of the proteolytic and esterolytic activities of porcine elastase, trypsins (bovine, ovine and porcine), subtilisins (Carlsberg and BPN') or the fungal proteinase K, which readily hydrolyses the chymotryptic substrate, α-*N*-benzoyl-L-tyrosine ethyl ester, was observed even with a 10-fold excess of inhibitor. The chymotryptic and tryptic activities of Pronase (*Streptomyces griseus* protease) were unaffected by the inhibitor.

Fig. 1 shows the profiles for the inhibition of bovine α-chymotrypsin and bovine chymotrypsin B. The magnitude of inhibition of the caseinolytic activity of both enzymes is the same as the inhibition of the esterolytic activity. Furthermore, Fig. 1 shows that the interaction of the inhibitor with bovine chymotrypsin B is different to that with bovine α-chymotrypsin. As reported previously [1] and confirmed here, the inhibition of α-chymotrypsin at [I] > 2[E] is not quantitative and substrate-induced dissociation of the inhibitor-α-chymotrypsin complex is observed. On the other hand, the inhibition of bovine chymotrypsin B, a closely related homologous enzyme, is quantitative (Fig. 1b)

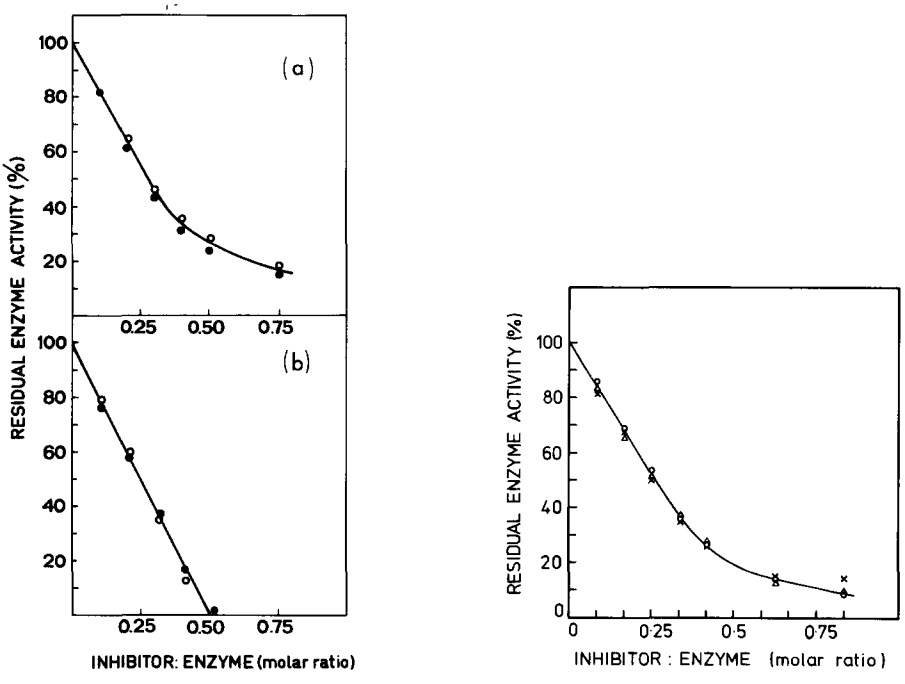

Fig. 1. (a) Inhibition of bovine α-chymotrypsin (62 μg) and (b) bovine chymotrypsin B (69 μg) by increasing amounts of winged bean chymotrypsin inhibitor at pH 8.0. Chymotryptic activity was assayed with α-*N*-benzoyl-L-tyrosine ethyl ester (●——●) and casein (○——○) as substrates, respectively.

Fig. 2. Inhibition of bovine α-chymotrypsin (75 μg; X——X), γ-chymotrypsin (92 μg; ○——○) and δ-chymotrypsin (92 μg; △——△) by increasing amounts of winged bean chymotrypsin inhibitor at pH 8.0. Residual chymotryptic activity was assayed with α-*N*-benzoyl-L-tyrosine ethyl ester as substrate.

with no evidence of substrate-induced dissociation of the inhibitor-chymotrypsin B complex. The apparent dissociation constant for the inhibition of chymotrypsin B was calculated according to Green and Work [8] and a value of $4 \cdot 10^{-11}$ M was obtained, assuming two identical reactive inhibitory sites. The apparent dissociation constant for the reaction with α-chymotrypsin was about $1.25 \cdot 10^{-8}$ M.

Inhibition of the other chymotrypsins examined was analogous. The different products of the activation of bovine chymotrypsinogen A, namely γ-chymotrypsin, δ-chymotrypsin and β-chymotrypsin and ovine α-chymotrypsin showed the same interaction profile with the inhibitor as found for bovine α-chymotrypsin (Fig. 2). Ovine chymotrypsin B and porcine chymotrypsin A were inhibited quantitatively in the same manner as bovine chymotrypsin B. Inhibition of the chymotrypsins was obtained over the pH range 5.0—9.0. In this pH range the time taken for complex formation between inhibitor and enzyme was too rapid (less than 1 min) to be observed under the normal assay conditions used. The stoichiometry of inhibition with all the chymotrypsins was in the molar ratio of 1 : 2, consistent with one molecule of inhibitor binding two molecules of enzyme.

Thus, not only is the known specificity of the inhibitor restricted to the chymotrypsins but it can distinguish between such closely related enzymes as bovine chymotrypsins α and B. As the serine proteases, including the chymotrypsins, have extensive binding sites for macromolecular substrates and inhibitors [9,10] the differences found in the apparent binding constants with chymotrypsins α and B, indicate that the inhibitor reactive site structure may recognize subtle topological differences at the binding sites of these two chymotrypsins. The inhibitor, therefore, should be a useful tool in comparing the specificity sites of chymotrypsins from other species.

At pH 8.0, where the inhibitor-chymotrypsin inhibition profiles were determined, bovine α-chymotrypsin is a cation, chymotrypsin B an anion and the inhibitor (p*I* approx. 6.4) an anion. To examine the effect of net charge on complex formation, inhibition profiles were determined at pH 5.0, 7.0 and 9.0 and the data are shown in Fig. 3. For chymotrypsin B, the profiles are essentially independent of pH. At pH 5.0, chymotrypsin B is close to its isoelectric point and an anion at pH 7.0 and 9.0 while the inhibitor is a cation at pH 5.0 and an anion at pH 7.0 and 9.0. This suggests that the formation of the inhibitor-chymotrypsin B complex is independent of net charges and ionization of functional groups on either the inhibitor or enzyme. However, in the case of α-chymotrypsin, the inhibition profiles show that the dissociation constant of the complex at pH 5.0 and 9.0 increases; at pH 5.0 the inhibitor and α-chymotrypsin are both cationic while at 9.0 both are anionic. Thus, while the net charge influences the dissociation of the inhibitor-α-chymotrypsin complex, the same type of inhibition profile ('non-stoichiometric') is observed irrespective of the net charge on the proteins. Thus, the ionization of groups on either inhibitor or enzyme does not appear to account for the differences in the interaction of the inhibitor with chymotrypsins α and B.

Inhibitor-chymotrypsin complex formation and isolation

The interaction of the inhibitor with the chymotrypsins under varying condi-

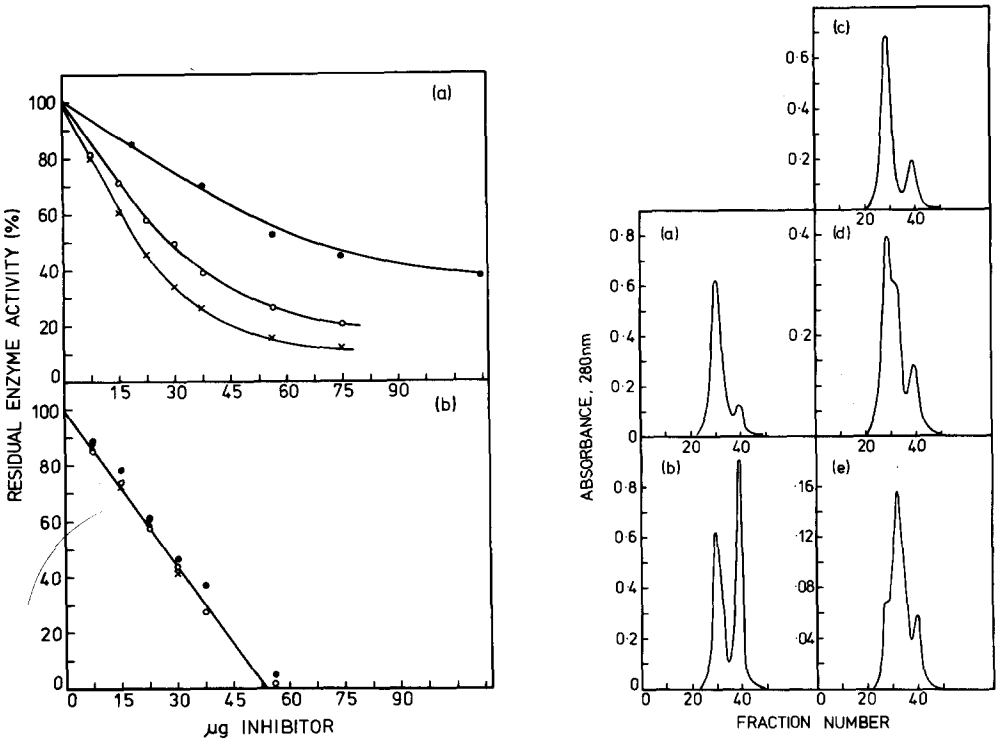

Fig. 3. (a) Inhibition of bovine α-chymotrypsin (89 μg) and (b) bovine chymotrpysin B (130 μg) by increasing amounts of winged bean chymotrypsin at pH 5.0 (●———●), pH 7.0 (X———X) and pH 9.0 (○———○). Residual chymotryptic activity was assayed with α-*N*-benzoyl-L-tyrosine ethyl ester as substrate at each pH value. The buffers used were 0.05 M citrate/0.01 M $CaCl_2$, pH 5.0, 0.05 M Tris-HCl/0.01 M $CaCl_2$, pH 7.0 and 0.05 M Tris-HCl, pH 9.0.

Fig. 4. Separation of mixtures of inhibitor and bovine chymotrypsin (α and B) on Sephadex G-100 (150 × 1 cm). The column was equilibrated with 0.05 M Tris-HCl/0.1 M NaCl, pH 8.0; fraction volume was 2.1 ml. (a) inhibitor (3.0 mg) plus α-chymotrypsin (2.5 mg); (b) inhibitor (1.67 mg) plus α-chymotrypsin (6.4 mg); (c) inhibitor (1.67 mg) plus chymotrypsin B (3.8 mg); (d) inhibitor (3.3 mg) plus chymotrypsin B (3.8 mg); (e) inhibitor (1.04 mg) plus chymotrypsin B (1.2 mg) ([I] ≃ [E]).

tions was examined by gel filtration on Sephadex G-100. Fig. 4 shows the elution profiles for mixtures of the inhibitor and α-chymotrypsin at pH 8.0 with the inhibitor (Fig. 4a) or the enzyme (Fig. 4b) present in excess. In addition to the inhibitor or chymotrypsin peak (M_r 22 000—25 000), a protein peak corresponding to a molecular weight of approx. 70 000 was obtained consistent with the formation of a 1 : 2 molar inhibitor-α-chymotrypsin complex.

Fig. 4 also shows the elution profiles for the complex formation with chymotrypsin B at pH 8.0. With an excess of chymotrypsin B, the inhibitor reactive sites are saturated and a 1 : 2 molar complex (M_r approx. 70 000) is formed (Fig. 4c). However, with an excess of inhibitor a shoulder (M_r approx. 48 000) was observed in addition to the major peak of M_r approx. 70 000 (Fig. 4d). This result indicates that chymotrypsin B reacts with the inhibitor to form a 1 : 1 molar complex as well as the 1 : 2 molar complex. Indeed when the inhibitor was mixed with chymotrypsin B in equimolar amounts the 1 : 1 molar

complex was the predominant species observed on gel filtration (Fig. 4e). In contrast, α-chymotrypsin formed a 1 : 2 molar complex at all concentrations of inhibitor to enzyme. These findings suggest that chymotrypsin B first binds at one reactive site of the inhibitor and then at the second reactive site. On this basis one may speculate that the two reactive sites of the inhibitor are not identical and that the specificity binding site of chymotrypsin B can distinguish between the two reactive sites, whereas that of α-chymotrypsin cannot. This suggests that the structural features defining the specificity binding sites of these two enzymes are not identical.

The formation of a 1 : 1 molar complex was also observed with ovine chymotrypsin B and porcine chymotrypsin A with [I] > [E]; ovine α-chymotrypsin, however, only yielded the 1 : 2 molar complex as found for the bovine enzyme. Gel filtration experiments at pH 5.0 and 9.0 confirmed the inhibition data and showed that chymotrypsins α and B formed a 1 : 2 molar inhibitor-chymotrypsin complex (with [E] > [I]). Those proteases which were not inhibited failed to interact with the inhibitor to form a higher molecular weight complex. The fact that the winged bean chymotrypsin inhibitor does not bind to elastase, subtilisin or *S. griseus* proteases A or B (the chymotrypsin-like enzymes of Pronase) highlights the narrow specificity of the inhibitor. A comparison of the structure of α-chymotrypsin and these proteases shows that the geometrical arrangements of the active site residues is similar. Even though they show limited identity of primary structure their tertiary structures are topologically equivalent. The primary specificity site S_1 [11] of bovine α-chymotrypsin is a well defined hydrophobic cleft adjacent to the active site residue, Ser-195 [12]. In elastase, for example, the cleft is also present but is partially occluded by a side chain (Val-216) which is absent in the chymotrypsins. Furthermore, the bottom of the cleft is partly filled by the side chain of Thr-226, again absent in the chymotrypsins [13]. These extra side chains make it sterically impossible for bulky aromatic side chains to bind in this cavity. These changes in the specificity site of elastase direct the specificity towards uncharged non-aromatic amino acids, such as alanine [13], and are sufficient to prevent the interaction with the winged bean chymotrypsin inhibitor. This suggests that an aromatic residue(s) may be involved in the reactive sites of the inhibitor. The architecture of the specificity site of the proteases also appears to be of importance in determining the interaction of the inhibitor. The primary specificity site of *S. griseus* protease B [14], for example, has been shown to be a surface depression containing two extra residues rather than the well defined cleft of α-chymotrypsin. These changes result in the primary specificity of Phe > Tyr > Leu compared with Tyr > Trp > Phe for α-chymotrypsin [15]. The fact that the inhibitor does not bind at this 'chymotryptic-like' specificity site suggests a strict geometry of binding between the inhibitor and the chymotrypsins. Studies to elucidate the structures of the reactive sites of the winged bean chymotrypsin inhibitor are being undertaken.

Stability of the inhibitor

No effect on the activity of the inhibitor was found in the pH range 2.0—11.5 (Fig. 5a). However, at pH values greater than 11.5 a rapid and irreversible decrease in the inhibitory activity was evident (Fig. 5b). It seems likely that

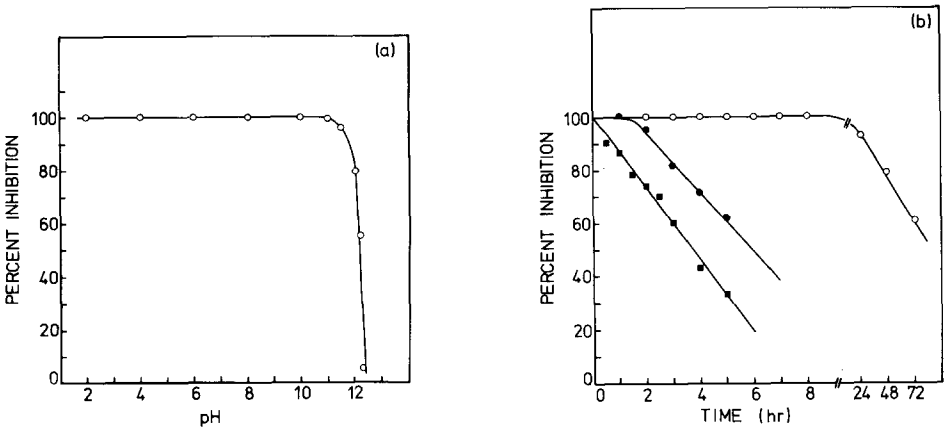

Fig. 5. (a) Effect of pH on the stability of the winged bean chymotrypsin inhibitor. (b) Stability of the inhibitor at high pH values. ○———○, pH 11.5; ●———●, pH 11.9; ■———■, pH 12.2. Experimental details described in Materials and Methods.

this irreversible loss of activity results from a conformational change in the inhibitor which affects the chymotrypsin inhibitory sites.

The stability of the inhibitor to heat is shown in Fig. 6. No inhibitory activity was lost when the inhibitor was heated to 90°C at pH 3.00 (0.001 M HCl); at pH 4.1 and 8.0 the inhibitor was stable only to approx. 70°C and then the inhibitory activity rapidly decreased upon further heating.

The inhibitor was rather resistant against denaturing agents (Table I), a characteristic common to most plant proteinase inhibitors. The inhibitor was stable in 8 M urea at 20°C while at 37°C a 57% loss of inhibitory activity was found in 24 h. The inhibitory activity was not affected by 0.12 M β-mercaptoethanol; however, a 10-fold increase of the reducing agent (1.2 M) resulted in a remarkable decrease in the inhibitory activity with a third of the activity being lost in 1 h at 20°C, while at 37°C inactivation was complete in 1 h. Reduction

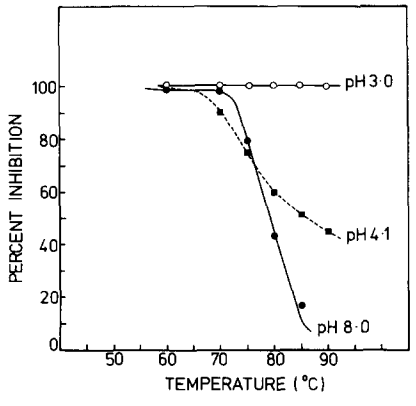

Fig. 6. Stability of the winged bean chymotrypsin inhibitor to heat treatment at different pH values. Experimental details described in Materials and Methods.

TABLE I

STABILITY OF THE CHYMOTRYPSIN INHIBITOR

The inhibitor activity towards bovine α-chymotrypsin was determined using *N*-benzoyl-L-tyrosine ethyl ester as substrate. The decrease in inhibitor activity was calculated with respect to a control solution of the inhibitor in 0.1 M Tris-HCl/0.01 M $CaCl_2$, pH 8.0. Inhibitor solutions treated with urea or β-mercaptoethanol, or both, were diluted 5.5-fold with the assay buffer. Controls containing an equal amount of urea/β-mercaptoethanol did not affect the activity of the chymotrypsin in the assays.

Treatment	Time (h)	Decrease of inhibitor activity (%)
Urea, 8 M, pH 8.0		
20°C	1	0
20°C	24	0
37°C	1	5
37°C	24	57.6
Urea, 8 M, pH 4.0		
20°C	1	0
37°C	1	7.3
β-Mercaptoethanol, pH 8.0		
0.12 M		
20°C	1	0
20°C	24	0
37°C	1.5	8.1
1.2 M		
20°C	1	33.7
37°C	1	100
β-Mercaptoethanol (0.12 M) urea (8 M), pH 8.0		
20°C	1	32.4
20°C	24	88.0
37°C	1	48.3
37°C	24	96.2

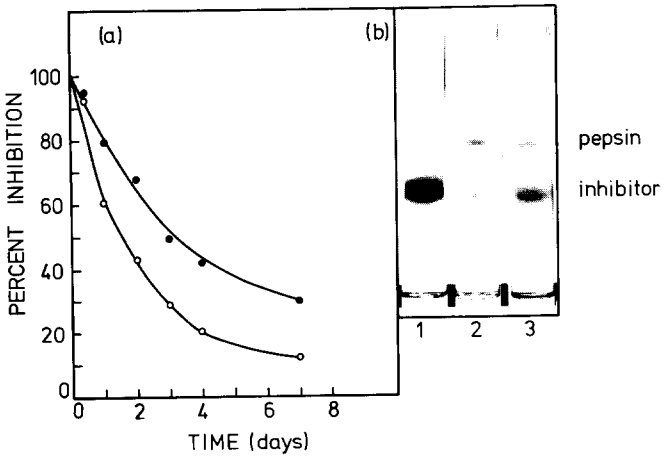

Fig. 7. (a) The effect of pepsin digestion on the inhibitory activity of the winged bean chymotrypsin inhibitor. 0.3 mg inhibitor digested with 0.12 mg pepsin (●———●) and 0.24 mg pepsin (○———○) at pH 2.0. 50-μl aliquots of the digest were taken at the intervals indicated and assayed for inhibitory activity. (b) SDS-polyacrylamide gel electrophoresis of the peptic digest at day 7. A 50 μl aliquot of digest was treated with 50 μl 2% SDS/2% β-mercaptoethanol. (1) inhibitor control (no pepsin) (2) inhibitor + 0.24 mg pepsin (3) inhibitor + 0.12 mg pepsin.

with 0.12 M β-mercaptoethanol and 8 M urea resulted in a complete loss of inhibitory activity only after 24 h at 37°C (Table I). The stability experiments show that the winged bean chymotrypsin inhibitor is a relatively stable protein even though it only has two disulfide bonds per molecule (M_r 21 000). In contrast, the high stability of the low molecular weight plant proteinase inhibitors (M_r approx. 8000) has been attributed to the high number of disulfide bonds and compact nature of these molecules [2]. The resistance of the disulfide bonds of the winged bean chymotrypsin inhibitor to reduction by 0.12 M β-mercaptoethanol, even in 8 M urea, suggests that the disulfide bonds are buried in the molecule and are inaccessible to solvent molecules. The fact that reduction abolishes inhibitory activity indicates that the disulfide bonds play an important role in maintaining the structural integrity and hence stability of the inhibitor.

Treatment of the inhibitor with catalytic amounts (1—2%, w/w) of porcine pepsin at pH 2.0 did not affect the inhibitory activity or degrade the inhibitor. To obtain digestion of the inhibitor, a pepsin to inhibitor ratio of 0.4 or 0.8 to 1.0 was required and even then the inhibitor was only slowly degraded (Fig. 7a). Even after 7 days digestion some inhibitory activity remained. SDS-polyacrylamide gel electrophoresis of the digest at day 7 showed that the inhibitor was being completely degraded with no evidence of smaller fragments being formed (Fig. 7b). The resistance of the inhibitor to pepsin degradation suggests that the chymotrypsin inhibitor may play a role in the toxicity of the winged bean and limit its utilization in the uncooked state. Raw winged beans are reported to be toxic to rats [16].

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