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Remarkable Phylum Selectivity of a *Schistocerca gregaria* Trypsin Inhibitor: The Possible Role of Enzyme–Inhibitor Flexibility

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A 35-mer polypeptide isolated from the hemolymph of desert locust *Schistocerca gregaria* (SG) proved to be a canonical inhibitor of bovine trypsin (K_i = 0.2 μM). Despite having a trypsin-specific arginine at the primary specificity P_1 site, it inhibits bovine chymotrypsin almost as well (K_i = 2 μM). Furthermore, while the latter reactivity improves 10^4-fold by the single replacement of P_1 Arg by Leu, changing P_1' from Lys to Met only moderately improves trypsin affinity (K_i = 30 nM). The apparent low compatibility to trypsin, however, is not observed *vs* two arthropodal trypsins: SG peptides with P_1 Arg inhibit crayfish and shrimp trypsins with K_i values in the picomolar range. This unprecedented high discrimination between orthologous enzymes is postulated to derive from flexibility differences in the protein–protein interaction. The more than four orders of magnitude phylum selectivity makes these peptides prospective candidates for agricultural use.

Key Words: **orthologous enzymes; serine proteases; insecticides; endogenous inhibitors; FT-IR; conformational flexibility; hydrogen exchange.**

Serine proteases with different specificity govern many biological functions. Their *in vivo* activity is usually controlled by endogenous protein protease inhibitors. Of the latter, the so-called standard mechanism (1), or canonical protease inhibitors (2) all share a distinct conformation of the active-site loop that binds to the protease (2, 3) and determines enzyme specificity. Based on differences in their size and global three-dimensional structure, they can be divided into at least 18 structurally nonhomologous families (3). To one of the most recently recognized serine protease inhibitor family, named the "grasshopper" family (3), belong a series of 35–40 amino acid long, single-chain polypeptides isolated from desert locusts *Locusta migratoria* (4–6) and *Schistocerca gregaria* (SG)[2] (7–9). The high similarity of their primary structures, and above all the conserved location of the cysteine residues that form the disulfide bridges, has already suggested very similar tertiary structures. Two-dimensional ^{1}H NMR spectroscopy of two members of this family, PMP-C and PMP-D2 (10–12), established that they share a common globular fold of a short three-stranded β-sheet. Their interaction with the target protease appears to be confined to five amino acids in their contact loops. Consequently, they are a particularly useful system for studying structure-function relationships in the process of molecular recognition, since only a few amino acids need be engineered to change enzyme–ligand interactions.

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[2] Abbreviations used: AMC, 4-aminomethylcoumarin; BPTI, bovine pancreatic trypsin inhibitor; FT-IR, Fourier-transform infrared; H/D exchange, hydrogen–deuterium exchange; NMR, nuclear magnetic resonance; pNA, *p*-nitroanilide; SG, *Schistocerca gregaria;* SGCI, *Schistocerca gregaria* chymotrypsin inhibitor; SGTI, *Schistocerca gregaria* trypsin inhibitor; Suc, succinyl; TCEP, Tris-2-carboxyethyl-phosphine.HCl; TFA, trifluoroacetic acid; Z, *N*-benzyloxycarbonyl.

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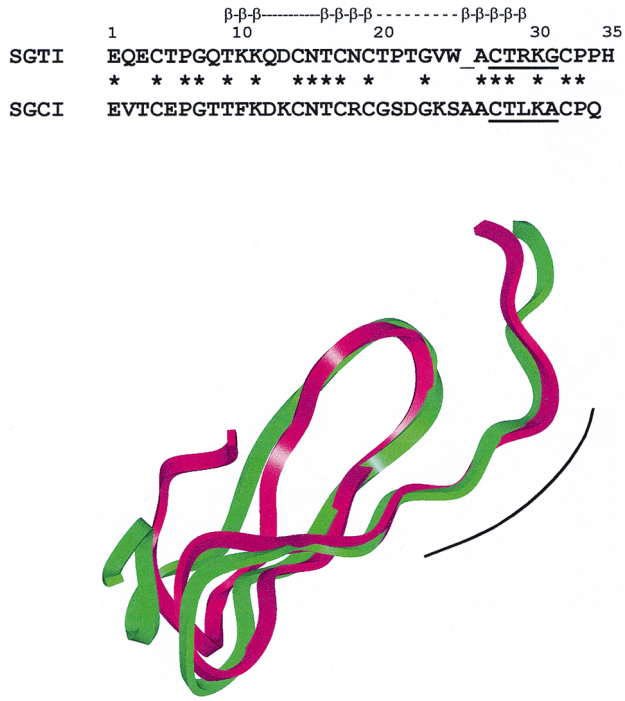

FIG. 1. Sequence alignment and NMR-structures of SGTI and SGCI. Conserved amino acids in the sequences are marked by asterisks. β-Strands are indicated. Active-site loop residues are underlined in the sequences. The backbone structure of SGCI (magenta) is the average of 20 NMR structures and the structure of SGTI (green) is the average of six structures. The protease binding region is indicated with a black line.

In a previous paper we reported the isolation and characterization of two inhibitor peptides from *S. gregaria* (9). When tested against bovine trypsin and chymotrypsin, representatives of two major specificity class in the chymotrypsin family of serine proteases, one proved to be a very potent and selective inhibitor of bovine chymotrypsin, and the other a much weaker and less selective inhibitor with some preference for trypsin. They were accordingly named SGCI and SGTI, for *S. gregaria* chymotrypsin and trypsin inhibitor, respectively. They display a significant sequence similarity and their almost completed NMR structures (Z. Gáspári, A. Patthy, L. Gráf, and A. Perczel, manuscript in preparation) revealed a high degree of structural similarity also (Fig. 1). The enzyme selectivity of the two SG inhibitors qualitatively corresponds to the arginine/leucine difference at P_1 [nomenclature according to Schechter and Berger (13)]. However, SGTI, which contains arginine at the P_1 position, generally the best P_1 residue for trypsin, is only a moderate inhibitor of bovine trypsin [(9) and Table II]. This is surprising in light of their rather high overall similarity, particularly the almost complete identity of their inhibitory loop sequences. In a search for the molecular basis of this phenomenon, P_1–P_1' site variants of SGTI were synthesized, and their equilibrium inhibitory constants (K_i) on bovine chymotrypsin and trypsin, and arthropodal trypsins were determined. We found that SGTI inhibited arthropodal trypsins with five orders of magnitude higher activity than bovine trypsin. To reveal the possible structural background of the phylum specificity we observed, the secondary structure and the conformational flexibility of the SG peptides were compared using FT-IR spectroscopy and hydrogen–deuterium exchange.

MATERIALS AND METHODS

Enzymes. Bovine trypsin was obtained from Cooper Biomedical, bovine chymotrypsin from Worthington (UK). Crayfish trypsin (*Astacus fluviatilis*) was a gift from Dr. R. Zwilling (Heidelberg, Germany), shrimp trypsin (*Penaeus monodon*) from Dr. Inn-Ho Tsai (Taipei, Taiwan). Both preparations appeared to be homogenous by SDS gel electrophoresis under reducing conditions. Their molecular masses were estimated to be 25,000 and 29,000 Da, respectively. The N-terminal amino acid sequences of crayfish and shrimp trypsins were determined by a pulsed-liquid-phase ABI 471A protein sequencer and found to be, respectively, the following: IVGGTDAVLG- and IVGGSEVTPG-.

Enzyme assays. Active enzyme concentrations were determined by active-site titration with the 4-methylumbelliferyl derivatives of *p*-guanidinobenzoate and *p*-trimethylammonium cinnamate chloride. The *p*-nitroanilide derivatives of benzoyl-L-arginine, Suc-Ala-Ala-Pro-Phe, Suc-Gly-Gly-Phe, Z-Gly-Pro-Arg, and Ac-Phe were obtained from Sigma (Hungary), and their hydrolysis was followed at 405 nm. The fluorescent substrates, 7-amino-4-methylcoumarin derivatives of Suc-Ala-Ala-Pro-Tyr and Suc-Ala-Ala-Pro-Arg, were synthesized as described earlier (14) and AMC production was measured at excitation and emission wavelengths 366 and 440 nm, respectively.

Inhibitors. Solid-phase synthesis of peptides and their homologues was performed on an ABI 431A peptide synthesizer using the standard Fmoc chemistry. Purification was carried out as described previously (9). Bovine pancreatic trypsin inhibitor (BPTI) was obtained from Sigma (Hungary).

FT-IR spectroscopy and H/D exchange. Kinetics of the H/D exchange of SGTI and SGCI in D_2O were measured by FT-IR spectroscopy on a Bruker IFS-28 FT-IR photometer using the procedure described earlier (15–17). Measurements were carried out at 25 ± 0.1°C as measured with a sensor attached directly to the CaF_2 cell windows. Aliquots of buffers were lyophilized and redissolved in D_2O immediately before starting the measurements. The H/D exchange experiments were carried out at pH values 8.0, 6.6, and 3.5. These values are the pHs of the buffer solutions in water before lyophilization of the samples. The respective p*D* values of the D_2O solutions were ~0.55 units higher. After scanning the baseline, lyophilized SGTI and SGCI samples (0.5 mg) were dissolved in the D_2O buffer solutions. The time of the addition of the buffer was taken as the start of the exchange. IR spectra (in the 400- to 4000-cm^{-1} region) were then recorded at regular intervals, the first at 30–40 s after dissolution of the peptides. A spectral resolution of 2 cm^{-1} was used. The number of scans used for recording (i.e., the time of recording a spectrum) was adjusted to the speed of the exchange (four scan measurements at the beginning and 128 scans toward the end). The band at 1450 cm^{-1} reflects the increasing number of ND groups and HDO molecules. Decrease of the amide II band reflects the H/D exchange of the amide protons in D_2O. For kinetic analysis, the fraction of the unexchanged protons, *X*, was plotted vs time. *X* was calculated from the amide II/amide I ratios as described earlier (17).

TABLE I

Steady-State Parameters for Reactions of Arthropodal Trypsins with Assay Substrates[a]

	Trypsin					
	Shrimp	Crayfish	Shrimp	Crayfish	Shrimp	Crayfish
Substrate	k_{cat} [s^{-1}]		K_M [M]		k_{cat}/K_M [M^{-1} s^{-1}]	
Z-Gly-Pro-Arg-pNA	n.d.[b]	280	n.d.	2.8×10^{-5}	n.d.	1.0×10^{7}
Suc-Ala-Ala-Pro-Arg-AMC	108	147	1.0×10^{-5}	2.0×10^{-5}	1×10^{7}	7.4×10^{6}

[a] The experiments were carried out at 25°C in 50 mM Tris, 300 mM KCl, 10 mM $CaCl_2$, pH 8.0.
[b] n.d., not determined.

Disulfide reduction and oxidation experiments. Completely oxidized pure inhibitors were dissolved in water (1 mg/ml). To 10 μl of this solution an equal volume of TCEP solution in water (1.65 mg/ml) was added and the reaction mixtures incubated at 45°C. After 0, 2, 5, 10, 20, 60, and 120 min incubation the reaction was terminated by adding 50 μl 0.1% TFA in water and the samples were injected onto an RP-HPLC column (Aquapore OD-300; 220 × 4.6 mm, Applied Biosystems, U.S.A.). Completely reduced samples of SGTI and SGCI were dissolved in 0.065 M Tris, pH 8.5 in 0.1% (w/v) concentration. Oxidation was achieved by stirring at room temperature in uncovered flasks. Aliquots of 20 μl were withdrawn at regular intervals and injected onto an RP-HPLC column.

Inhibition kinetics. Inhibition kinetics for the peptides were measured essentially by the method of Empie and Laskowski (18) as described in detail previously (9). Enzyme and inhibitor were incubated in stoichiometric amounts in concentrations of at least an order of magnitude higher than the K_i expected and assayed for remaining activity at regular intervals. The time when no more decrease in activity was observed was accepted as sufficient to reach apparent equilibrium for the enzyme–inhibitor association. Plotting activity loss vs time allowed the concomitant rough determination of k_{on}. When incubation time was approximately 24 h, activity loss without inhibitor was checked in independent experiments and was shown to be negligible.

K values were determined by nonlinear fitting of data by computer (19) to Eq. [1].

$$\frac{[E]}{[E]_0} = 1 - \frac{[E]_0 + [I]_0 + K - \{([E]_0 + [I]_0 + K)^2 - 4[E]_0[I]_0\}^{1/2}}{2[E]_0} \quad [1]$$

Assay substrates were chosen to yield maximum sensitivity in the respective enzyme concentration range. For trypsin, they were Z-Arg-pNA, Z-Gly-Pro-Arg-pNA, and Suc-Ala-Ala-Pro-Arg-AMC; for chymotrypsin, Ac-Phe-pNA and Suc-Ala-Ala-Pro-Phe-pNA. From K and k_{on}, k_{off} values could be calculated, and, if necessary (practically at K values higher than 10^{-8} M), competition by substrate during the assay time was taken into account by using Eq. [2].

$$K_i = K/(1 + [S]/K_M) \quad [2]$$

K_M values used for assay substrates with bovine trypsin are data from our laboratory published earlier (14, 20), those for crayfish and shrimp trypsins were determined in the present work (Table I).

RESULTS

Inhibition of Bovine Trypsin and Chymotrypsin: The Effect of Changing P_1 and P_1' in SGTI

In a previous paper we reported that by changing P_1 Leu and P_1' Lys residues in SGCI to Arg and Met, respectively, the SGCI-L30R/K31M variant was almost as potent and selective for inhibition of trypsin as the natural SGCI peptide was for inhibition of chymotrypsin (9). To gain further information on inhibitor–enzyme interactions, here we performed changes in SGTI to obtain variants with the same active-site peptide bond as in SGCI, and determined their inhibitory constants.

TABLE II

Equilibrium K_i Values of SGTI and Its Analogues *vs* Bovine Trypsin and Chymotrypsin[a]

Peptide	Reactive site peptide bond (P_1–P_1')	K_i [M] vs Trypsin	K_i [M] vs Chymotrypsin	$lg \frac{K_{i,chymotrypsin}}{K_{i,trypsin}}$
SGTI	Arg-Lys	$2.1 \pm 0.4 \times 10^{-7}$[b]	$2.0 \pm 0.7 \times 10^{-6}$[b]	0.98
SGTI-K30M	Arg-Met	$3.0 \pm 0.3 \times 10^{-8}$	$7.1 \pm 1.6 \times 10^{-6}$	2.32
SGTI-R29L	Leu-Lys	$1.0 \pm 0.3 \times 10^{-5}$	$3.5 \pm 1.6 \times 10^{-10}$	−4.46

[a] Experiments were carried out at 25°C in 50 mM Tris, 300 mM KCl, 10 mM $CaCl_2$, pH 8.0. Values are means ± SD of at least three independent experiments.
[b] From Ref. (9).

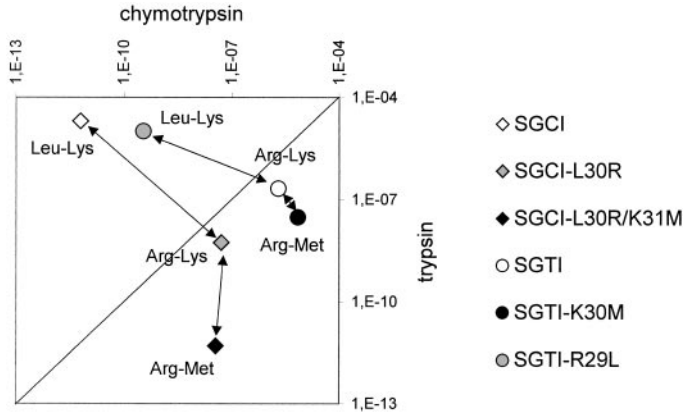

FIG. 2. Inhibition of bovine chymotrypsin and trypsin by SGTI (○) and SGCI (▽) peptide variants. Ordinate shows K_i vs trypsin, abscissa K_i vs chymotrypsin. The reactive-site P_1–P_1' residues of the inhibitors are also displayed. Arrows indicate single replacements; diagonal shows nonselective inhibition.

Data in Table II show that replacement of the trypsin-specific Arg with chymotrypsin-specific Leu at the P_1 primary specificity site of SGTI resulted in an increase of almost four orders of magnitude in chymotrypsin and a 50-fold decrease in trypsin inhibition. Consequently, as apparent from the last column of Table II, this single replacement reversed and improved inhibitor selectivity: starting with SGTI, a moderately selective trypsin inhibitor, SGTI-R29L became a rather potent and selective chymotrypsin inhibitor. This peptide is close in both affinity and selectivity to native SGCI (Fig. 2).

In an attempt to improve trypsin affinity of SGTI, we replaced P_1' lysine by methionine, which makes a better fit with the enzyme according to data of both acyl transfer reactions (21, 22) and substrate hydrolysis (23, 24). Indeed, this change converted SGTI into an inhibitor with an increased trypsin and decreased chymotrypsin affinity in agreement, even in quantitative terms, with the above mentioned data. However, the extent of the effect of the P_1' site change is basically different from that with SGCI (Fig. 2), where the same Lys to Met change led to a three orders of magnitude increase of trypsin inhibition. Compared to the SGCI peptide with the Arg-Met reactive-site bond, the SGTI peptide (SGTI-K30M) is not only less selective than SGCI-L30R/K31M, but, more importantly, is a weaker inhibitor of bovine trypsin by four orders of magnitude (Fig. 2).

Disulfide Reduction and Oxidation Experiments

Reduction of the disulfide bonds in SGTI was much slower than in SGCI. Based on the HPLC profiles, 50% conversion was reached after 20 min with SGTI, and after 5 min with SGCI (Fig. 3, left).

When the reduced SG peptides were allowed to reoxidize and refold, we observed a difference in the time needed for disulfide bond formation by HPLC analysis. In a systematic study of oxidation (an indicator of folding), of SGTI, we observed a single intermediate leading to the formation of the native structure, which was complete within 90 min. In the case of SGCI, several intermediate forms were seen, and the process was not yet complete in 120 min (Fig. 3, right). That both reduction is faster and oxidation slower in SGCI indicates a looser structure for this SG peptide compared to SGTI.

Infrared Spectroscopy and Hydrogen–Deuterium Exchange

The flexibility of protein molecules is reflected in perpetual conformational fluctuations. These reversible, noncooperative local rearrangements expose buried segments of the polypeptide chain to the solvent. In D_2O, H/D exchange occurs during such exposure.

H/D exchange revealed differences in conformational flexibility of the SG peptides. The exchange rates of the amide protons were followed by FT-IR spectroscopy. Figure 4 shows the original infrared spectra as well as their change with incubation time in D_2O. The amide I band of the infrared spectra of protein molecules primarily arises from the C═O stretching vibration of the amide-groups of the polypeptide backbone and is very sensitive to the secondary structures and conformational changes (25, 26). SGCI and SGTI display very similar amide I bands, both showing an unusually sharp double maximum. The peak at 1637 cm^{-1} indicates the high β-structure content, while the peak at 1670 cm^{-1} shows mainly the turn content. The spectra undoubtedly indicate that in this aspect, SGCI and SGTI secondary structures are practically identical, as also indicated by the NMR data.

Decrease of the amide II band reflects the H/D exchange of the amide protons in D_2O (Fig. 4). H/D exchange experiments were carried out at pH values 8.0, 6.6, and 3.5. At pH 8.0 more than 90% of the amide protons were exchanged during the dead time of the measurements (data not shown). These high exchange rates are due to the small size and the relatively small hydrophobic cores of the molecules as well as to the strong pH dependence of the amide proton exchange rates.

At pH 6.6 almost 80 and 60% of the amide protons were exchanged within the dead time of the measurements for SGCI and SGTI, respectively. The time course of the exchange of the remaining fractions (Fig. 5A) indicates a several-fold faster reaction for amide protons in SGCI than for those in SGTI. A similar difference is seen at pH 3.5 where, due to the small chemical exchange rate of the exposed amide protons,

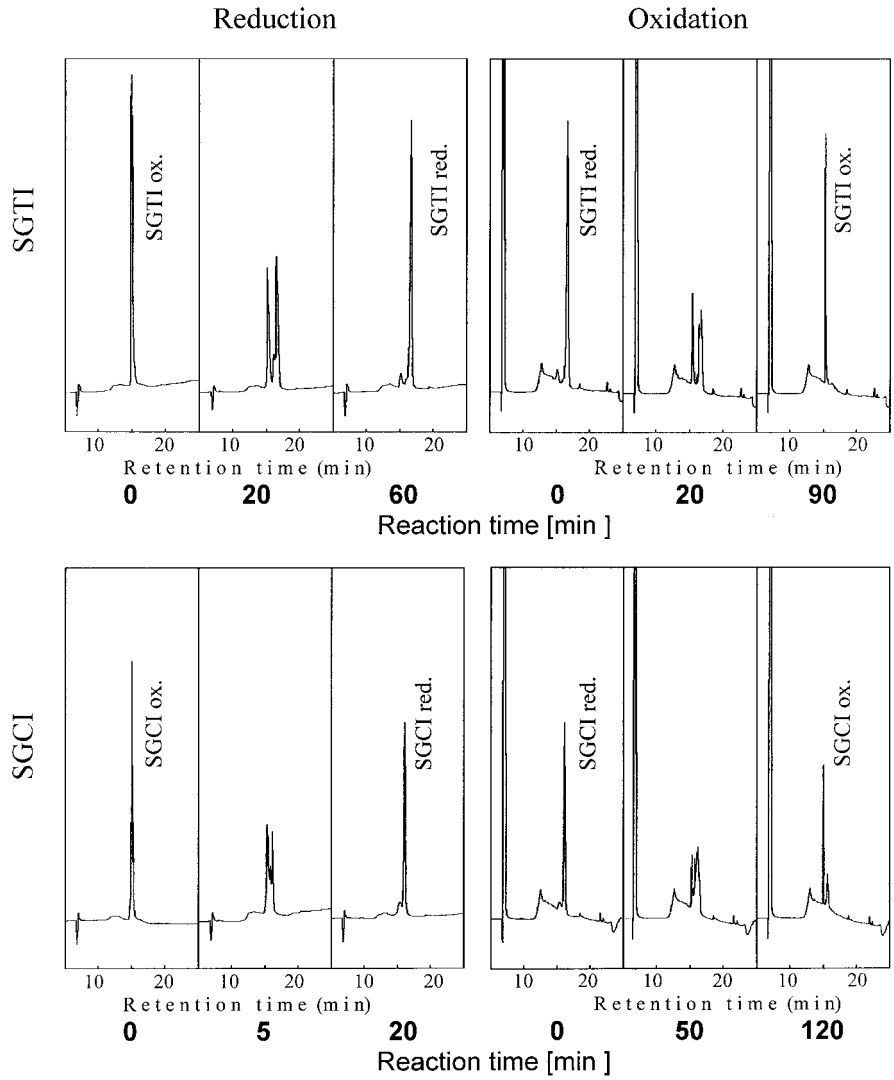

FIG. 3. HPLC profiles of samples withdrawn at indicated times from reduction (left) and oxidation (right) of SGTI (upper) and SGCI (lower). Conditions: flow rate, 1 ml/min; gradient, 0–100% B in 30 min (A, 0.1% TFA in water; B, 0.08% TFA in 80% acetonitrile/20% water). Detection at 220 nm. The middle part of each panel represents approximately 50% conversion.

the time course of the exchange of more than half of all protons could be followed (Fig. 5B). In the first part of the curves there is no significant difference between the rates of exchange, which can be explained by the fact that the exchange at this pH is slow enough to see the exposed amide protons on the surface of the molecules. The rates for the next part of the curves which corresponds to the buried protons differ by approximately an order of magnitude.

These results show that despite the similar secondary structure, the conformational flexibility of the core of SGTI is significantly less than that of SGCI.

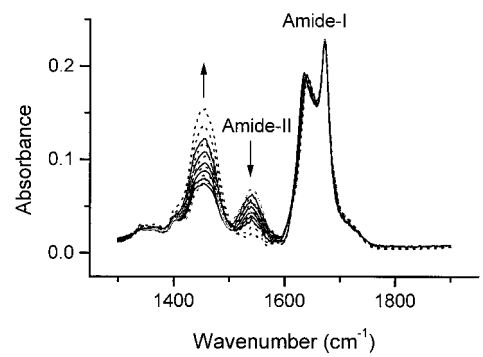

FIG. 4. Infrared spectrum sets of the H/D exchange experiments on SG peptides in 20 mM citrate, pH 3.5, at 25°C. SGTI, solid line; SGCI, dotted line. Arrows show directions of changes. The amide II band shows the decreasing number of amide protons. Amide I band indicates the similar secondary structure of the two molecules.

Inhibition of Arthropodal Trypsins

If flexibility is a factor that significantly influences the reaction of SG peptides with their target enzymes,

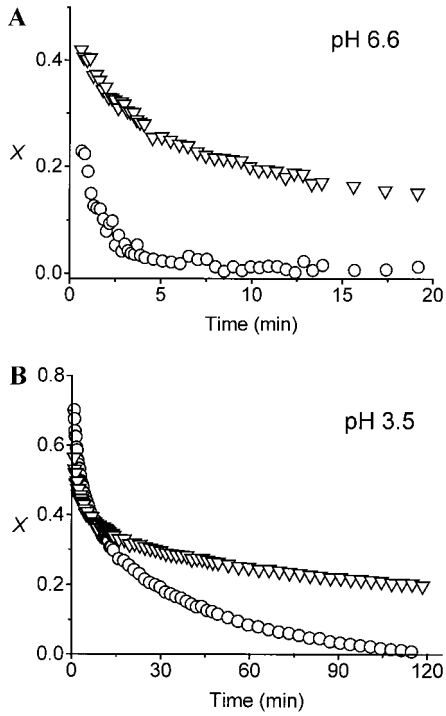

FIG. 5. H/D exchange data of SGTI (▽) and SGCI (○) at pH 6.6 (A) and at pH 3.5 (B). *X* is the fraction of the unexchanged amide protons.

this should also manifest in reactions where the enzyme partners are different in this respect. Trypsins from lower organisms, as a rule, have significantly fewer disulfide bridges than the mammalian enzymes, that allows for a greater overall structural flexibility of the proteins. When tested against trypsins from crayfish and shrimp, we found that all four SG peptides that have a trypsin-specific Arg at P_1, are very strong inhibitors of both arthropodal trypsins (Table III). The inhibitory dissociation constant K_i values of SGTI, its variant SGTI-K30M as well as the SGCI variants *vs* crayfish and shrimp trypsins are all in the picomolar range, i.e., in the range of the best bovine trypsin inhibitor BPTI (Table III). Although the K_i values in the order of 10^{-12} are at the extreme edge of this experimental approach, the use of highly sensitive fluorescent substrates allowed reliable determinations in this range. Typical inhibition curves obtained for BPTI *vs* bovine, and SGTI *vs* the crayfish trypsin are shown in Fig. 6. It is also apparent from Table III that, in contrast to BPTI, all SG peptides show a preference for the arthropodal trypsins. This preference, however, varies between a factor of 4 and 70,000.

DISCUSSION

Inhibition of Bovine Trypsin and Chymotrypsin by SG Peptides with the Same P_1–P_1'

Canonical inhibitors of serine proteases contact the enzyme active sites by four to five residues of their binding loops. The selectivity toward trypsin/chymotrypsin is basically determined by their P_1 residue, although P_1' also contributes. Figure 2 shows that the effects on trypsin and chymotrypsin inhibition of changing only the reactive-site P_1 and P_1' residues of SGTI and that of SGCI conforms to the above premise. Figure 2 also shows that identical changes affect selectivity in a similar way, but the resulting SGTI peptides are significantly weaker and less selective inhibitors than the SGCI ones with the same reactive site. The difference is more prominent in trypsin inhibition: SGTI(R29L) with Leu-Lys is almost as strong and as selective an inhibitor as native SGCI, whereas the SGCI-derived L30R/K31M is a much better inhibitor of trypsin than SGTI-K30M with the same reactive-site peptide bond.

The difference in trypsin inhibition between the latter two peptides is mainly due to difference in effects of

TABLE III

Equilibrium K_i Values of the *Schistocerca gregaria* Peptides with Arginine at P_1 and BPTI vs Bovine and Arthropodal Trypsins[a]

Peptide	Reactive site peptide bond (P_1–P_1')	K_i [M] vs Bovine	K_i [M] vs Crayfish (trypsin)	K_i [M] vs Shrimp	$\frac{K_{i,bovine}}{K_{i,crayfish}}$
SGTI	Arg-Lys	$2.1 \pm 0.4 \times 10^{-7}$[b]	$3.0 \pm 0.7 \times 10^{-12}$	$1.4 \pm 0.1 \times 10^{-12}$	70,000
SGCI-L30R	Arg-Lys	$5.5 \pm 1.5 \times 10^{-9}$[b]	$2.0 \pm 0.3 \times 10^{-12}$	$3.8 \pm 0.2 \times 10^{-13}$	2,800
SGTI-K30M	Arg-Met	$3.0 \pm 0.3 \times 10^{-8}$	$5.0 \pm 0.2 \times 10^{-12}$	$1.0 \pm 0.1 \times 10^{-12}$	6,000
SGCI-L30R/K31M	Arg-Met	$5.0 \pm 0.3 \times 10^{-12}$[b]	$1.2 \pm 0.4 \times 10^{-12}$	$5.5 \pm 0.7 \times 10^{-12}$	4
BPTI	Lys-Ala	$6.7 \pm 0.5 \times 10^{-13}$	$2.7 \pm 0.6 \times 10^{-12}$	n.d.	0.25

[a] Experiments were carried out at 25°C in 50 mM Tris, 300 mM KCl, 10 mM $CaCl_2$, pH 8.0. Values are means ± SD of at least three independent experiments.

[b] From Ref. (9).

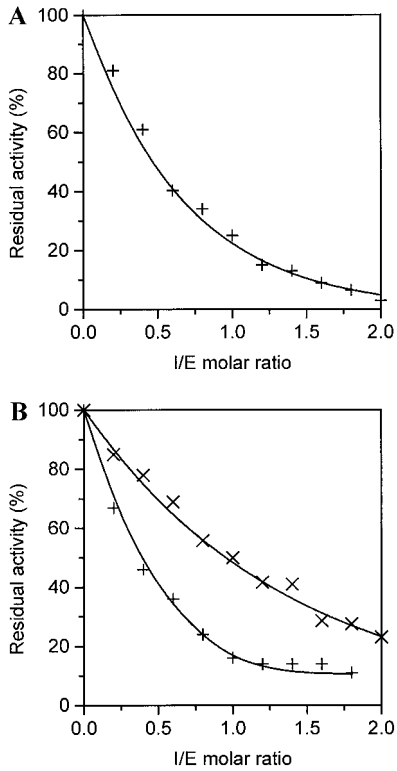

FIG. 6. Typical inhibition curves, obtained in 50 mM Tris–HCl, 20 mM $CaCl_2$ at pH 8.0, 25°C. Ordinate shows residual enzyme activity in percentage, abscissa I/E molar ratios. (A) Bovine trypsin/BPTI system; concentration of enzyme was 1×10^{-11}. (B) Crayfish trypsin/SGTI system; concentration of enzyme was 1×10^{-11} M (X) and 1.7×10^{-11} M (+).

changes at P_1'. The Lys/Met change altered the affinity of the SGTI peptide as expected on the basis of S_1' preferences of trypsin and chymotrypsin (23, 24): K_i vs trypsin decreased and that vs chymotrypsin increased. The extent of the change, i.e., less than 10-fold, compares well with the results obtained by acyl transfer studies (23, 24), but is significantly smaller than in the case of SGCI (see Fig. 2). Apparently the SGCI scaffold allows for a better fit to trypsin than the SGTI one.

The differences in their reactions, particularly with trypsin, between SGTI and SGCI seem remarkable in the light of their structural similarity. In the inhibitory, protease binding region in the C-terminal segment of the molecule from P_2 to P_2', except the Arg/Leu difference at the primary specificity site the two only differ at P_2' (Gly vs Ala). It is not too likely that the latter accounts for the superiority of SGCI and its reactive-site variants over SGTI and its reactive-site variants in both affinity and selectivity.

On the other hand, our hydrogen–deuterium exchange experiments indicated a higher overall conformational flexibility of SGCI compared to that of SGTI. This is in line with the results of earlier NMR studies on SGCI analogue PMP-C and SGTI analogue PMP-D2 (10–12). Such a difference between the synthetic SG peptides is also supported by our present results on the disruption and reformation of the disulfide bridges. Both reduction is faster and oxidation is slower with SGCI than with SGTI, indicating a looser tertiary structure for the previous one.

Inhibition of Different Trypsins by SG Peptides

If difference in flexibility may influence inhibitor–enzyme reactions, then a difference is also expected in reactions of one inhibitor with enzymes of different flexibilities. Trypsins from lower organisms have significantly fewer disulfide bridges and may accordingly have looser structures than mammalian ones. This initiated the studies of crayfish and shrimp trypsin inhibition. These studies revealed that all SG peptides with trypsin-specific Arg at P_1 are almost uniformly very potent inhibitors of the invertebrate trypsins. The widely different increase of affinity (Table III) diminishes the several orders of magnitude differences between SGTI and SGCI peptides with same P_1–P_1' against bovine trypsin: the difference is only fivefold between them against the arthropodal trypsins. The higher affinity interaction with invertebrate trypsins indicates that some feature of these enzymes is basically different from that of the mammalian one. The possibility of differential exosite binding also arises. The general notion is, however, that contact is restricted to the P_2–P_2' segment. Thus we compared enzyme sequences forming the corresponding S_2–S_2' sites. An alignment of bovine and crayfish (27) sequences reveals, and analysis of a partial sequence of shrimp (28) confirms that the S_1' sites bear negative charge in the arthropodal enzymes, in contrast to the positive charge of the bovine enzyme. Figure 7 shows the charges in the segments forming the S_1' site [Loop 40 and Loop 60 (22)]. In the reaction of natural SGTI with a positively charged Lys at P_1', the repulsion with bovine trypsin may turn into an attraction with crayfish trypsin. The effect of the Met/Lys exchange is in accordance (Tables II and III) with the above reasoning, but the fact that SGTI-K30M with a neutral residue at the P_1' site is not a much better inhibitor of bovine trypsin than SGTI (Table II), suggests that this electrostatic effect in itself can hardly account for the striking difference.

Bovine	N-----SGYHF 34 39	CY**K**SGIQ 58 64	**+1**
Crayfish	Q**E**TFLGFSFHF –	CVYG**DD**G – –	**-3**
Shrimp	**E**G----V**E**SHF – –	n.d.	**-2**

FIG. 7. Alignment of trypsin sequences that form S_1' site in the bovine enzyme. Charged residues are bold; the sum of charges is displayed after each sequence.

Another factor that may influence the interaction can be flexibility. The above-mentioned common feature of arthropodal trypsins, that they contain fewer disulfide bridges, might make them more flexible compared to the vertebrate ones (29). The lack of Cys22–Cys157 interdomain bridge that was recently shown to play a key role in the domain-domain stabilization of vertebrate trypsins (29) may in itself cause this difference. That the melting temperature of crayfish trypsin as measured by differential scanning calorimetry was found to be 61.2°C, 11°C lower than that of rat trypsin (29 and J. Kardos, P. Závodszky, and L. Gráf, unpublished data] might also indicate a higher overall flexibility. Since partners should conform to each other for an optimal fit, it seems plausible that the rather rigid SGTI can induce a better fit with the more flexible arthropodal trypsin than with the more rigid bovine one.

Phylum Specificity of SGTI Peptides

Without structural data of the arthropodal enzymes and/or complexes with their inhibitors the structural features behind the very high orthologous specificity remains a mystery. The >10,000-fold preference for trypsins from lower organisms of a protein protease inhibitor is almost unprecedented. Usually the K_i values determined for trypsins from different species with the commonly used, and phylogenetically rather distant proteinaceous inhibitors such as BPTI and STI differ by less than 100-fold either from each other, or from those with bovine enzyme. Within this range K_i values vs the bovine enzyme are the lowest, with a few exceptions only (30, 31). Our own determinations of the K_i values for BPTI on bovine and crayfish trypsin (Table III) are in line with the above notion. Of the scarce systematic studies on cross-species reactivity, data on the affinity of bean trypsin inhibitors (32, 33) as well as of inhibitors of different origin (30, 31, 34) to trypsins and chymotrypsins from different vertebrate species indicate a variance of less than two orders of magnitude. Somewhat higher, approximately 300-fold difference in inhibition of human and porcine trypsins by a trypsin inhibitor from a nematode (35), and two orders of magnitude difference was reported between porcine and human elastase vs the Lys15Val homologue of BPTI (36).

As far as arthropodal enzymes are considered, shrimp trypsin was shown to have similar specificities toward inhibitors as the mammalian trypsins (28). We also found crayfish trypsin to be inhibited by BPTI only 6-fold less than the bovine enzyme (Table III). Even trypsin from *L. migratoria* (37) was reported to be inhibited to the same extent as bovine trypsin by four natural proteinaceous inhibitors, with the only exception of STI, that preferentially inhibited bovine trypsin.

From the aspect of a potential application as an insecticide, the *S. gregaria* peptides studied in our laboratory share the advantageous feature of being endogenous protease inhibitors of an insect, making it less probable that insect pests will develop resistance against them (38). However, the five orders of magnitude selectivity toward trypsins from lower organisms *vs* from mammalian ones makes SGTI the more promising candidate from which to develop an effective insecticide.

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