Serine Protease Inhibition by Insect Peptides Containing a Cysteine Knot and a Triple-stranded \( \beta \)-Sheet*

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Three insect peptides showing high sequence similarity and belonging to the same structural family incorporating a cysteine knot and a short three-stranded anti-parallel \( \beta \)-sheet were studied. Their inhibitory effect on two serine proteases (bovine \( \alpha \)-chymotrypsin and human leukocyte elastase) is reported. One of them, PMP-C, is a strong \( \alpha \)-chymotrypsin inhibitor (\( K_i = 0.2 \) \( \text{nm} \)) and interacts with leukocyte elastase with a \( K_i \) of 0.12 \( \mu \text{m} \). The other two peptides, PMP-D2 and HI, interact only weakly with \( \alpha \)-chymotrypsin and do not inhibit leukocyte elastase. Synthetic variants of these peptides were prepared by solid-phase synthesis, and their action toward serine proteases was evaluated. This enabled us to locate the P1 residues within the reactive sites (Leu-30 for PMP-C and Arg-29 for PMP-D2 and HI), and, interestingly, variants of PMP-D2 and HI were converted into powerful inhibitors of both \( \alpha \)-chymotrypsin and leukocyte elastase, the most potent elastase inhibitor obtained in this study having a \( K_i \) of 3 \( \text{nm} \).

In the last decade, naturally occurring serine protease inhibitors (1) have been the focus of many studies, mainly for two reasons: first, the target proteins control functions in a variety of fundamental proteolytic processes in humans and mammals (blood clotting, digestion, inflammation, fibrinolysis), in invertebrates such as insects (immune system, digestion, protection against their predators) or worms (protection against their host), and plants (protection against insect attack); second, low molecular weight inhibitors of serine proteases have been attractive tools for studying the general aspects of protein conformation and protein-protein interactions (2). In the present study, we report the inhibitory properties of three homologous peptides (primary and tertiary structures) toward \( \alpha \)-chymotrypsin, trypsin, and human leukocyte elastase.

We have previously isolated two peptides, PMP-C and PMP-D2, from the brain and the fat body of the insect Locusta migratoria (3). These peptides are composed of 36 and 35 residues, respectively, and are cross-linked by three disulfide bonds. The Thr-9 of PMP-C has an uncommon \( \gamma \)-glycosidic linkage to a single fucose moiety. There is 40% strict identity between PMP-C and PMP-D2 with conservation of the Cys positions (3). Moreover, they are located on the same peptide precursor, and, by Northern blot analysis, it has been shown that the gene encoding this precursor is mainly transcribed in the fat body (4). In this paper, we describe the isolation and characterization of HI, a novel locust peptide.

Because the isolation from insect extracts is time-consuming and yields only small amounts of peptides, we have prepared, at a reasonable scale (5-10 mg), by solid-phase synthesis, PMP-D2 (5) and PMP-C with and without the fucose moiety. Although they are small peptides with a high disulfide content, no sequence similarities could be found when comparing them with small toxins or small protease inhibitors. However, the milligram quantities of PMP-D2 obtained by solid-phase synthesis enabled us to study its tertiary structure by two-dimensional nuclear magnetic resonance, which showed interesting similarities with the tertiary fold of both \( \alpha \)-conotoxin GVIA, a calcium channel blocker, and the Ascaris chymotrypsin/elastase inhibitor (6). This prompted us to evaluate their protease inhibitory activity.

In the present paper, we report on the inhibitory activity of PMP-C, PMP-D2, and HI toward serine proteases (bovine \( \alpha \)-chymotrypsin, human leukocyte elastase, and porcine trypsin) using the natural and synthetic peptides. Since the P1 residue (7) within the reactive site of serine protease inhibitors determines the specificity for the cognate enzyme, mutational or synthetic changes of the P1 residue and/or replacement of active site residues should greatly influence both the specificity and the potency of the inhibition. For that reason, we have prepared by solid-phase synthesis variants of PMP-C, PMP-D2, and HI, in which one or two residues within the reactive site have been changed, and we have evaluated their inhibitory properties toward HLE, \( \alpha \)-chymotrypsin, and trypsin.

**EXPERIMENTAL PROCEDURES**

Materials

Chemicals

The acetonitrile, trifluoroacetic acid of HPLC quality, methyl tert-butyl ether, and dimethylformamide (DMF) were from SDS (Sètç, France). The N-ethylisodisopropylamine (DIEA) was obtained from Merck (Darmstadt, Germany). The Wang \((\text{p}-\text{benzyloxybenzyl alcohol})\) resins came from Novabiochem (Meudon, France), and \( N^p \)-Fmoc amino acids derivatives were purchased from Millipore (St. Quentin Yvelines, France).

1. H. Hietter, M. Schultz, and H. Kunz, submitted for publication.

2. We prefer using "variant" instead of "mutant" (which refers to a mutational change) since they are obtained by solid-phase synthesis.

3. The abbreviations used are: HLE, human leukocyte elastase; RP-HPLC, reverse phase-high performance liquid chromatography; Fmoc, N-(9-fluorenyl)methoxycarbonyl; DMF, dimethylformamide; DIEA, ethylisodisopropylamine; pNA, para-nitroanilide. It should be mentioned that PMP-C, PMP-D2, and HI are given names of the peptides, rather than abbreviations.
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The difference of 6 Da between the measured and calculated masses is accounted for by the presence of 3 disulfide bonds.

| Peptide       | Molecular mass | Calculated | Measured |
|---------------|----------------|------------|----------|
| PMP-C L30V    | 3765.34        |            |          |
| PMP-C K31M/A32G | 3769.37       |            |          |
| PMP-D R29L    | 3716.26        | 3709.63    | 0.10     |
| PMP-D R29L/K30M | 3719.29       | 3713.26    | 0.34     |
| HI R29L       | 3679.25        | 3673.02    | 0.85     |

Peptide concentrations mentioned in this article refer to the concentration of active enzymes. The Michaelis constants \( K_m \) were determined using standard procedures. Unless otherwise stated, all kinetic experiments were performed at 25 °C in 50 mM Tris containing 20 mM CaCl₂, pH 8.0, a solution that will be referred to as "the buffer" throughout the text.

Measurement of Equilibrium Dissociation Constant \( K_e - K \), was calculated from equilibrium titration experiments. 990-μl reaction mixtures containing constant amounts of enzyme and increasing amounts of inhibitor in the buffer were incubated at 25 °C for 15 min, an incubation time that was sufficient to ensure maximum enzyme inhibition under our experimental conditions. Reactions were started by the addition of 10 μl of substrate stock solution, and the release of p-nitroaniline was followed at 410 nm using a Unicon 941 spectrophotometer (Kontron) and recorded until the rate of substrate breakdown remained constant.

Measurements of association (\( K_{ass} \)) and dissociation (\( k_{diss} \)) rate constants were performed in the presence of HLE and chymotrypsin. Reaction mixtures were prepared with the same peptide concentration, were used. The release of p-nitroanilide was followed at 410 nm using a Cary 2200 spectrophotometer (Varian) on line in an IBM PS2 Model 30 microcomputer. Nonlinear regression analysis of progress curves was done using the Enzfitter software ( Biosoft, Cambridge, UK).
Amino acid sequence of the peptide named HI and comparison with the sequence of PMP-D2 (72% identity); B, sequence comparison between PMP-C and PMP-D2 (a gap is introduced to maximize the homology between the two sequences).

**RESULTS**

Isolation and Primary Structure Determination of a Novel Peptide, HI, from the Hemolymph of *L. migratoria*—While purifying from the hemolymph PMP-C and PMP-D2 as reference compounds, a novel peptide, named HI, was isolated. The sequencing of HI, carried out by automated Edman degradation, showed a 72% strict identity with PMP-D2 (Fig. 1). The molecular mass, measured by electrospray mass spectrometry, was 3716.43 Da. This value differs by 6 Da from the data obtained by Edman degradation (3722.28 Da), thus suggesting that the 6 Cys are all involved in 3 disulfide bonds. Moreover, because of its high homology with PMP-D2, it is likely that the disulfide pairing is the same as in PMP-D2 (6).

Protease Inhibition by Natural and Synthetic PMP-C, PMP-D2, and HI—Preliminary experiments have shown that only PMP-C inhibits HLE. By contrast, chymotrypsin was found to interact with the 3 peptides. Interestingly, none of the peptides was found to inhibit porcine trypsin in our experimental conditions.

Fig. 2 shows the effect of increasing quantities of synthetic PMP-D2 on constant quantities of chymotrypsin. Substrate was added to equilibrium mixtures of enzyme and inhibitor. The release of p-nitroanilide versus time was stable after 20–30 s indicating that E, I, S (enzyme, inhibitor and substrate, respectively), and their complexes have reached their equilibrium. Calculation of the best estimate of the substrate-dependent equilibrium constant $K_{i(app)}$ was performed by nonlinear regression analysis of the experimental data based on Equation 1 (13):

$$a = 1 - \frac{(E)_0 + (I)_0 + K_{i(app)}}{\sqrt{(E)_0 + (I)_0 + K_{i(app)}}^2 - 4(E)_0(I)_0}$$

where $a$, the enzymatic fractional activity, is the ratio of the velocity in the presence of inhibitor to that in its absence. The true $K_i$ (Table II) was deduced from $K_{i(app)}$ using the following relationship: $K_i = K_{i(app)}/(1 + [S]/K_m)$, where $[S]$ is the initial substrate concentration. The $K_i$ value for natural PMP-D2 was shown to be identical with that of the synthetic peptide (Table II).

Equilibrium dissociation constants governing the interactions between chymotrypsin and synthetic HI and between HLE and PMP-C (natural and synthetic nonfucosylated) were determined using similar equilibrium titration experiments (Table II).

Since PMP-C (natural and synthetic nonfucosylated) binds chymotrypsin very tightly, $K_i$ was obtained through $K_{diss}$ and $K_{diss}$. These parameters were determined using the progress curves method (13, 14). A typical curve illustrating chymotrypsin inhibition by nonfucosylated PMP-C is shown in the inset of Fig. 3A. The curve is biphasic, i.e., the pre-steady state release of product is followed by a steady state, confirming that PMP-C reversibly interacts with chymotrypsin. Since no significant decrease of the initial substrate concentration occurred during the progress of the reaction and, since $I_0 \geq 10 \times E_0$, product accumulation versus time can be described by the following equation:

$$P = v_xt + \frac{v_x}{k} (1 - e^{-kt})$$

where $P$ is the product concentration, $v_x$ is the rate of substrate hydrolysis at $t = 0$, $v_x$ is the steady state velocity, $k$, the apparent first order rate constant, $S$, the substrate concentration, $E$, the initial enzyme concentration, $I$, the initial inhibitor concentration, and $k$ is the rate constant. The linear increase of $k$ with increasing concentration of PMP-D2, the linear increase of $K_{i(app)}$ with increasing concentration of PMP-D2, and the linear increase of $k$ strongly suggests that no reaction intermediate accumulates (within the range of inhibitor concentrations used) and that $E$ and $I$ interact according to a simple bimolecular and reversible mechanism.

Fig. 3B shows the effect of the initial substrate concentration on $k$; the linear increase of $k$ with 1/F ($F = 1 + [S]/K_m$) indicates that inhibitor and substrate compete for the binding to the enzyme. Hence, $k$ and $I_0$ are related as follows (15):

$$k = k_{ass} (1 - [S]/K_m) + k_{diss}$$

where $K_m$ is the Michaelis constant, $k_{ass}$, the second order rate constant was calculated from the slope of the linear curve shown in Fig. 3A using $K_m = 23 \mu M$. $I_{diss}$, the first order dissociation rate constant, is given by the intercept of the curve with the ordinate. The values of $K_{diss}$, $k_{diss}$, and $K_i$ were determined from the fucosylated and nonfucosylated PMP-C shown in Table I; these kinetic constants are similar, indicating that the fucose moiety does not affect chymotrypsin inhibition.

The inhibitory properties (toward chymotrypsin and HLE) of the variants of PMP-C, PMP-D2, and HI were examined using similar methods, and the results are given in Table II. Determination of the P1 Amino Acid by Synthetic Replacement of the Amino Acid at the Presumed Site—It is well documented that a-chymotrypsin inhibitors usually have bulky and aromatic amino acid residues such as Tyr, Phe, Leu, or Met as their P1 residue, while elastases have moderately large hydro-
Effect toward both in PMP-C is Leu-30, which could account for its inhibitory phobic residues such as Leu, Met, Ala, Val as P1, but never Phe. Initially, mucous proteinase inhibitor (17) and Ascaris chymotrypsin inhibitors; the change of Leu to Val should have little if any effect on the tertiary fold of PMP-C; small peptidic inhibitors are supposed to react in a substrate-like manner, and Val is a P1 residue found in the substrates of HLE (16).

As shown in Table II, the L30V variant lost most of its ability to inhibit α-chymotrypsin. However, it retained its weak activity toward HLE (Table II). The spectacular change in the inhibition of α-chymotrypsin observed with the L30V variant indicated that it was possible to lose specifically the inhibitory activity toward chymotrypsin, while retaining the anti-elastase property. Therefore, we concluded that the changed amino acid Leu was the P1 residue, the decrease in affinity being due to an inappropriate P1 residue and not to the misfolding of the PMP-C variant.

Confirmation of the Determined P1 Residue by Conversion of PMP-D2 and H1 into Powerful α-Chymotrypsin Inhibitors—The comparison of PMP-C with PMP-D2 and H1 sequences enabled us to locate the reactive site of PMP-D2 and H1 by simply superimposing them: C28TLKAC33, PMP-C; C27TRKGC32, PMP-D2; C27TRKAC32, HI.

Thus, the most probable reactive sites P1-P9 of R29L in PMP-D2 and H1 are Arg-29-Lys-30. To confirm this hypothesis, the presumed P1 residues (Arg) were replaced by Leu in PMP-D2 and H1 in order to have the same reactive site Leu-Lys as in PMP-C. The potency of both R29L variants toward α-chymotrypsin inhibition was increased significantly, and they were both converted into HLE inhibitors. The PMP-D2 variant was found to be the strongest inhibitor of HLE obtained in this study (Table II).

When comparing the amino acids within the reactive site P3-P9 of PMP-C and the R29L variants of H1 and PMP-D2, one can notice the strict identity of the recognition site between PMP-C and the H1 variant (CTLKAC), whereas PMP-D2 variant has a Gly in the P2 position (CTLKG) instead of Ala; these results are indicative of an Ala in position P2 which may be preferable to Gly for α-chymotrypsin inhibition, while the contrary is true for elastase inhibition.

Design of a Better Elastase Inhibitor—Several elastase inhibitors have been reported to have a Met residue at P1; for instance, mucous proteinase inhibitor (17) and Ascaris chymo-

### Table II

| Peptide | Active site P1-P9 | α-Chymotrypsin | Elastase |
|---------|-------------------|----------------|----------|
|         | k_{\text{cat}} | k_{\text{dis}} | K_{i} | k_{\text{cat}} | k_{\text{dis}} | K_{i} |
| PMP-C (Natural) | C_{28}T-L-K-A-C_{33} | 8.00 ± 1.60 | 1.62 ± 0.04 | 0.20 ± 0.05 | 120 ± 5 |
| PMP-C (Synthetic) |  | 7.50 ± 0.75 | 1.00 ± 0.10 | 0.13 ± 0.03 | 180 ± 29 |
| PMP-C (L30V) | -V- | 265 ± 16 | 2.00 ± 0.02 | 45.70 ± 5.90 | 22.85 ± 3.20 |
| PMP-C (K31M/A32G) | -M-G- | 4.50 ± 0.40 | 0.74 ± 0.02 | 0.16 ± 0.02 | 1.30 ± 0.03 | 4.50 ± 0.70 | 3.50 ± 1.30 |
| PMP-D2 (Natural) | C_{27}T-R-K-G-C_{32} | 1500 ± 189 |  |  |  |
| PMP-D2 (Synthetic) |  | 1300 ± 65 | 1.30 ± 0.01 | 2.12 ± 0.24 | 2.12 ± 0.24 |
| PMP-D2 (R29L) | -L- | 1.40 ± 0.04 | 1.10 ± 0.30 | 0.78 ± 0.24 | 6.00 ± 0.17 | 0.68 ± 0.06 | 26.00 ± 2.60 | 38.20 ± 7.20 |
| PMP-D2 (K30M) | -L- | 1.03 ± 0.12 | 2.10 ± 0.40 | 2.04 ± 0.62 | 340 ± 58 |
| HI (Synthetic) | C_{27}T-R-K-A-C_{32} | 340 ± 58 |  |  |  |
| HI (R29L) | -L- | 12.00 ± 0.50 | 0.80 ± 0.17 | 9.07 ± 0.017 | 38.20 ± 7.20 |

* NI, no inhibition.

\[ \text{Equation 2} \]

\[ k_{\text{cat}} = \frac{[S]_{0}}{K_{m}} \]

\[ k_{\text{dis}} = \frac{[S]_{0}}{K_{m}} \]

\[ K_{i} = \frac{[S]_{0}}{K_{m}} \]

\[ \text{F}\text{. 3. A. effect of increasing concentrations of synthetic nonfucosylated PMP-C (0.14 to 0.72 um) on the apparent first order rate constant (k). The inset shows a progress curve for the inhibition of α-chymotrypsin: the substrate Suc-Ala₂-Pro-Phe-pNA (0.25 mw) and PMP-C (0.43 um) were allowed to equilibrate in the cuvette, and the reaction was initiated by the addition of α-chymotrypsin (10 mw, final concentration), the release of p-nitroanilide was followed for 6 min. The theoretical curve is generated using Equation 2 and the best estimate of k_B, effect of increasing concentrations of Suc-Ala₂-Pro-Phe-pNA (1/8) on the apparent first order rate constant (k) of the α-chymotrypsin-PMP-C complex (F = 1 + [S]_{0}/K_{m}).]
trypsin/elastase inhibitor (18) have Leu-Met as P1-P1' reactive site. Therefore, considering the strongest elastase inhibitor obtained in this study (PMP-D2 variant R29L), a double variant R29L/K30M (with Met as P') was designed in order to improve the binding to HLE. Indeed, the variant R29L/K30M is 4-fold stronger than the previous one with a \( K_i \) of 3 nM, confirming the importance of the Met in position P'.

To investigate the role of the residues beyond the P1-P1' bond, we have prepared a double variant of PMP-C: K31M/A32G (which has the same P3-P3', CTLMGC, as the most powerful elastase inhibitor R29L/K30M variant of PMP-D2) and examined its inhibitory activity; this variant is still 7- to 8-fold weaker HLE inhibitor than the double variant of PMP-D2 (a Met as P', in this case, does not increase the potency toward HLE). In contrast, the double variant of PMP-C is more effective toward \( \alpha \)-chymotrypsin than the PMP-D2 double variant. Thus, the residues beyond the P1-P1' bond seem to have an effect on the specificity toward proteases, and variants of PMP-D2 seem to be more specific toward elastases than the variants of PMP-C.

**DISCUSSION**

The results of this study clearly show that PMP-C and PMP-D2 differ significantly with respect to their selectivity toward serine proteases, even though they exhibit a high sequence homology (45%) and are structurally related.\(^4\) It seems reasonable to hypothesize that a common ancestor might have adapted for specific and diverse biological functions by punctual mutations that do not affect the overall three-dimensional structure. In that respect, it is remarkable that a unique substitution in the reactive site of PMP-D2 (from Arg to Leu) is sufficient to restore the serine protease inhibitory activity.

Since PMP-C is a tight-binding reversible inhibitor and has a small and compact shape and an exposed binding loop, we propose to include it in the large group of the "small canonical tight-binding serine protease inhibitors." This group of protease inhibitors consists of 16 different families and includes peptides ranging from 29 to 120 amino acids (2). Interestingly, the peptides of this group are structurally unrelated, but share some properties such as hydrophobic cores (often maintained by disulfide bonds), stability toward unfolding, and, more remarkably, an exposed binding loop (containing the scissile P1-P1' bond) that fits into the active site cleft of the serine protease. The specificity of serine protease inhibitors is significantly, but not exclusively, determined by the nature of the P1 residue in the reactive site. Although in most families of proteases, the active regions are highly conserved, in the serine protease inhibitors, there is no consensus sequence for the reactive site emerging yet. Indeed, retention of activity in these proteins exists even though the P1 residue has been changed (1). In some cases, substitutions lead to a predictable change in the inhibitory specificity (17, 19-21).

Taking into account the variability of the P1 region, we have designed "variants" of PMP-C, PMP-D2, and HI by targeting precisely the amino acid replacement. The aim of this study was to determine the P1 residue of the peptides and to increase the affinity toward HLE, since a variety of elastase inhibitors have been shown to be effective in animal models of emphysema, acute respiratory distress syndrome, rheumatoid arthritis, cystic fibrosis, bronchitis, or acute pancreatitis.

We have proved that Leu-30 is actually the P1 position by critically reading this manuscript, helpful comments, and his interest in this study. We wish to thank M. Schneider from the Laboratory of Dr. J. Hoffmann (URA 1490, CNRS, Strasbourg) for her precious help in collecting the hemolymph from the locusts. We also express our gratitude to O. Sorokine (URA 31, CNRS, Strasbourg) for the automated sequencing of the peptide named HI.

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