Analysis by phage display selection and site-directed retromutagenesis of the Mustard Trypsin Inhibitor 2 reactive site

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A R T I C L E   I N F O

Article history:
Received 26 February 2010
Received in revised form 27 May 2010
Accepted 27 May 2010

Keywords:
Circular dichroism
Helicoverpa zea
Phage display
Proteinase inhibitor

A B S T R A C T

The Mustard Trypsin Inhibitor (MSI) family is a small family of plant protease inhibitors so far only found in Brassicaceae. Using a phage display selection, MTI-2 (Mustard Trypsin Inhibitor 2) mutants were detected and analysed for their biochemical characteristics. Retromutants of the selected MTI-2 proteins were constructed and expressed in the Pichia pastoris system. The recombinant proteins were analysed by activity assays against bovine trypsin and Helicoverpa zea trypsin, and by circular dichroism. These analyses suggest a strict requirement for a specific proline residue adjacent to the inhibitor reactive site and give additional insights for future phage display application.

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Introduction

Protease inhibitors (PIs) are present in a wide variety of living organisms including microorganisms, plants, and animals. They act as regulators of endogenous proteolytic activity, as participants in many developmental processes and as host’s defense components. Because of their important role in living organisms, PIs have been extensively studied in order to allow a better understanding of their structural and functional properties.

Plant PIs polypeptides are generally present at high concentration in storage tissues (up to 10% of protein content), but also detectable in leaves in response to the attack of insects and pathogenic microorganisms (Ryan, 1990). PIs function by inhibiting proteases present in insect guts or secreted by microorganisms, causing a reduction in the availability of amino acids necessary for their growth and development (Lawrence and Koundal, 2002). Several transgenic plants expressing PIs have been produced in the last 16 years and tested for enhanced defensive capabilities against insect pests (Valueva and Mosolov, 2004). However insects have evolved ways to adapt to the ingestion of PIs, switching the protease arsenal of their guts (Volpicella et al., 2003). The co-evolution of insect proteases and plant inhibitors constitute another attractive field of investigations for scientists interested in research on plant PIs. The possibility of designing new PIs with higher or different activities is now being exploited (Ceci et al., 2003; Gruden et al., 2004; Volpicella et al., 2006).

The PI studied in this paper is the Mustard Trypsin Inhibitor II, MTI-2, a potent inhibitor of trypsin isolated from mustard seeds (Menegatti et al., 1992), which belongs to a family of PIs (MSI family) so far restricted to the Brassicaceae (De Leo et al., 2002). The mti-2 gene has been completely characterized (Ceci et al., 1995). The function of MTI-2 in plant defense mechanisms has been extensively investigated either in planta using transgenic tobacco and Arabidopsis thaliana plants (De Leo et al., 2001, 1998), or in vitro using the MTI-2 recombinant protein (Ceci et al., 1995; Volpicella et al., 2000).

Similar protease inhibitors have been fully characterized in rapeseed (Brassica napus) seeds (Ceciliani et al., 1994) and more recently similar genes have been identified in A. thaliana (atti-1/6) (Lin et al., 1999) in A. lirata (alti-1/4) (Clauss and Mitchell-Olds, 2004), in rapeseed (rti-1/3) (De Leo et al., 2006) and in two wild Brassicaceae (Volpicella et al., 2009).

Up to now only the tertiary structure of the precursor form of the A. thaliana trypsin inhibitor (ATTp GenBank accession number 246816) has been reported for the MSI family (Zhao et al., 2002), showing one α-helix (residue 31–39) and an antiparallel β-sheet in a β-hairpin conformation which consist of two β-strands (residues 45–48 and 58–61) connected by a type IV β-turn (residues 52–55). The putative reactive site loop is solvent-exposed, with the P1 residue (Arg27) pointing outward to the solvent.

Using the phage display approach, a library of MTI-2 inhibitor variants was created by randomization of a stretch of five consecutive codons in the reactive site (Ceci et al., 2003). The efficiency of this library was demonstrated against the bovine trypsin and chymotrypsin, allowing the identification of novel MTI-2 derived antitrypsin and antichymotrypsin inhibitors (Ceci et al., 2003). Only
recently a trypsin-like protease, insensitive to the MTI-2 activity, was purified from the gut of *Helicoverpa zea* larvae that were raised on an inhibitor-containing diet (Volpicella et al., 2003). Repeated selection rounds against the insensitive *H. zea* protease allowed the identification of MTI-2 derived mutants. Unfortunately mutants showed to be ineffective in inhibiting both *H. zea* insensitive and bovine trypsins. We started from this observation to further investigate by site-directed mutagenesis and biochemical and biophysical analysis the role of amino acids located in the region of the reactive site of MTI-2.

### Materials and methods

#### Materials

XI-1 blue (Stratagene) and ElectroMAX DH12S cells (Life Technologies) were used as bacterial host. GS115 (*his4*P. pastoris) strain was obtained from Invitrogen. Unless specifically indicated, all DNA manipulations were according to standard procedures or as specifically indicated in the manuals for the Quickchange site-directed mutagenesis kit (Stratagene); expression in *P. pastoris* (Invitrogen), Pwo DNA polymerase (Boehringer). All recombinant plasmids were checked by sequencing. Bovine trypsin (TPCK treated, chymotrypsin free) and chymotrypsin (TLCK treated, trypsin free) were purchased from Sigma.

#### Oligonucleotides

The following primers (Invitrogen) were used in the amplifications for cloning in the pPIC9 yeast vector. Triplets correspond to amino acid codons: restriction sites are underlined.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTI2-XhoI 5′-AAAAAAAAA CTC GAG AAA AGA GAG GCT GAA GCT GAT AGC GAG TGC CTG AAA GAA TAC-3′</td>
<td>restriction site XhoI.</td>
</tr>
<tr>
<td>MTI2-UGA 5′-GGGGGGAAT TCA CTG ATC AAA AGG GCT GTC GTT GTA AAT G-3′</td>
<td>restriction site EcoRI.</td>
</tr>
</tbody>
</table>

Positions of MTI2-XhoI and MTI2-UGA primers in the mitochondrial gene *mti-2* were as already described (Volpicella et al., 2000).

The following primers (Invitrogen) were used for the retromutations amplifications. Triplets correspond to amino acid codons. The modified amino acid codons in the selected phage display mutant are in bold.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1P,F 5′-GG TTC CCT TCT TGC AAG CCT CGG CCT CTG CCG AGC-3′</td>
<td>1P,R 5′-CG TCG GCG AAA GCC GAG GCT TGC AGA AAG GGA AGC-3′</td>
</tr>
<tr>
<td>2AP,F 5′-GG TTC CCT TCT TGC GCG CCT CGG CCT TCT GCG AGC-3′</td>
<td>2AP,R 5′-CG TCG GCG AAA GCC GAG GCG CGC AGA AAG GGA AGC-3′</td>
</tr>
</tbody>
</table>

#### Expression and activity of MTI-2 mutants

Selected MTI-2 variants were expressed in the yeast *P. pastoris* as already described for the native MTI-2 (Volpicella et al., 2000). Expression, purification and characterization of recombinant proteins were also according to reported procedures (Volpicella et al., 2000).

Activities against trypsin and chymotrypsin were determined by using the substrates 2-Arz-Arz-p-nitroanilide (RRpNA) and N-succinyl-L-Ala-L-Ala-L-Pro-L-Leu-p-nitroanilide (SAAPLPNA), respectively, as described (Volpicella et al., 2001, 2000).

#### Selection of the MTI-2 phage display library

Phage particles were obtained as already described (Ceci et al., 2003). Selection was carried out with protease immobilized on microtiter plates essentially as described by Griffiths et al. (1994). Microplates were pretreated with 200 μl of a 20 mM Tris–HCl pH 7.8, 0.25% glutaraldehyde solution for 45 min. Bovine trypsin or *H. zea* protease were coated on microtiter plates (MaxiSorp Nunc-Immuno Plate) by using 30 μl trypsin solution (0.2 mg ml⁻¹ in 20 mM Tris–HCl pH 7.8) or *H. zea* protease solution (about 0.01 mg ml⁻¹ in 20 mM Tris–HCl pH 7.8). Binding of protease and selection procedure was performed as already described in Ceci et al. (2003).

#### Analysis of the MTI-2 mutants by circular dichroism

CD spectra were recorded at different time intervals on 10 μM protein solutions (in 20 mM phosphate buffer, pH 7) using a Jasco J-810 spectropolarimeter (Jasco Inc., Easton, MD, USA), a quartz cuvette with a 1 mm optical path, a wavelength interval of 185–250 nm, and a 0.1 nm data pitch. Each spectrum corresponds to an average of 10 scans and was baseline corrected and then smoothed by applying adjacent averaging or an FFT filter. The ellipticity is reported as mean residue molar ellipticity (deg cm² dmol⁻¹) according to $\theta = 100 \cdot [\theta] / (C \cdot L \cdot N)$, where $[\theta]$ is the observed ellipticity in degrees, C is the molar concentration of the peptide, L is the optical path length (in cm), and N is the number of amino acid residues in the protein (N = 63). Quantitative estimations of the secondary structure content were made by using the DICROPROT software package (Deleage and Geourjon, 1993).

### Results

#### Selection and analysis of MTI-2 variants against *H. zea* protease

The MTI-2 phage display library was used to select MTI-2 variants against an insensitive *H. zea* trypsin (HzTrypsin-S) (Volpicella et al., 2003). Because of limited available amounts of that protein, a selection on microplates was developed instead of the previously reported selection in immunoaffines (Ceci et al., 2003). As control, a selection against bovine trypsin was done. Phage particles recovered from the fourth cycle of both selections were analysed in more detail. For bovine trypsin selection, DNA from 14 different colonies was sequenced (Table 1a); for HzTrypsin-S, DNA from 19 different colonies was sequenced (Table 1b).

From an analysis of the sequences of the 14 trypsin selected phages, it can be seen that the sequence APRIF, corresponding to the wild-type, is highly represented (13 times) (Table 1a). The HzTrypsin-S selected variants are not dominated by a particular sequence (Table 1b). Only in the Hz1 (SQKMH), Hz2 (PKRL*), Hz13 (KNRLS), Hz15 (SHRQM) and Hz17 (QGRPA) mutants, the basic residues in P1 position (K and R) correspond to the expected residues for a trypsin inhibitor (Laskowski and Kato, 1980). Because of the low quantity of pure HzTrypsin-S, it was not possible evaluate the capacity to bind clonal phage stocks to immobilized HzTrypsin-S by phage ELISA.

#### Characterization of MTI-2 variants

MTI-2 variants Hz1, Hz13, Hz15 and Hz17 were produced in the yeast *P. pastoris*, purified and characterized in terms of their apparent equilibrium constants (Laskowski and Kato, 1980) against HzTrypsin-S. Unexpectedly, all the selected variants did not inhibit HzTrypsin-S at all (data not shown).

Also the activity against bovine chymotrypsin of the Hz7 mutant, with a W residue in P1 position, was evaluated and found negligible (not shown).

Circular dichroism analysis of the Hz13 mutant was also carried out. Results are shown in Fig. 1 together with data obtained for wild-type MTI-2. Fitting data of CD spectra of the wild-type MTI-2 and mutant KNRLS show a reduction of the α-helical content in KNRLS compared to MTI-2, resulting in an increase of random coil regions.
Table 1
Protein sequence of selected MTI-2 phage variants selected against bovine trypsin (a) and *H. zea* protease (b).

<table>
<thead>
<tr>
<th>Phage clone</th>
<th>Protein sequence</th>
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<tbody>
<tr>
<td>(a)</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>APRIF</td>
</tr>
<tr>
<td>Try1-13</td>
<td>APRIF</td>
</tr>
<tr>
<td>Try14</td>
<td>APRFM1</td>
</tr>
<tr>
<td>(b)</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>APRIF</td>
</tr>
<tr>
<td>Hz1</td>
<td>SQKMH</td>
</tr>
<tr>
<td>Hz2</td>
<td>PKRL*</td>
</tr>
<tr>
<td>Hz3</td>
<td>LSPPL</td>
</tr>
<tr>
<td>Hz4</td>
<td>HQARA</td>
</tr>
<tr>
<td>Hz5</td>
<td>WS*HA</td>
</tr>
<tr>
<td>Hz6</td>
<td>TTNLNS</td>
</tr>
<tr>
<td>Hz7</td>
<td>NHWLT</td>
</tr>
<tr>
<td>Hz8</td>
<td>PPMPQ</td>
</tr>
<tr>
<td>Hz9</td>
<td>TPLTA</td>
</tr>
<tr>
<td>Hz10</td>
<td>QQNQA</td>
</tr>
<tr>
<td>Hz11</td>
<td>RRSHT</td>
</tr>
<tr>
<td>Hz12</td>
<td>MVCVST</td>
</tr>
<tr>
<td>Hz13</td>
<td>KNRLS</td>
</tr>
<tr>
<td>Hz14</td>
<td>LHTPW</td>
</tr>
<tr>
<td>Hz15</td>
<td>SHRQM</td>
</tr>
<tr>
<td>Hz16</td>
<td>LRNWNP</td>
</tr>
<tr>
<td>Hz17</td>
<td>QGRPA</td>
</tr>
<tr>
<td>Hz18</td>
<td>LSCR*</td>
</tr>
<tr>
<td>Hz19</td>
<td>NITTE</td>
</tr>
</tbody>
</table>

WT: phage particles carrying recombinant mustard protease inhibitor MTI-2; *TAG stop codon, coding for Gln in supE E. coli cells.

To test the possibility to recover for the protein a proper folding, experiments of denaturation/renaturation of KNRLS were performed, without obtaining any success (data not shown) (Fig. 2).

Site-directed retromutation of Hz13 and characterization of obtained inhibitors

In order to study the influence of single amino acids on the activity of MTI-2 mutants, experiments of retromutation were performed on the Hz13 (KNRLS) mutant using the Stratagene's Quick Change II XL Site-Directed Mutagenesis Kit (see Section "Materials and methods"). The strategy of site-directed mutagenesis is shown in Fig. 3.

From the KNRLS clone, two retromutants were produced: KPRLS with the replacement of one amino acid near the reactive site (N > P); APRLS in which the substitution takes place for two amino acids (KN > AP).

MTI-2 retromutants were produced in *P. pastoris*, purified (Fig. 4) and characterized in terms of their apparent equilibrium constants (Laskowski and Kato, 1980) against bovine trypsin and *HzTrypsin-S* (Table 2). Beside the recovered capacity to inhibit bovine trypsin, the two mutants show CD spectra similar to that of the wild-type molecule (Fig. 1).

Discussion

Results concerning the activity of MTI-2 mutants in the region of the reactive loop are reported in this study. Mutants obtained both by phage display selection of a large combinatorial library of the inhibitor against *HzTrypsin-S*, and by site-directed retromutation of a specific phage display selected mutant were considered.

At the moment the structural features leading to the adaptation of insect proteases are not completely understood. They may be explained assuming either that modifications around the active site pocket impair proper interaction with the inhibitor, or as a direct consequence of specific modifications of protease active site (Bayes et al., 2005; Lopes et al., 2004, 2006; Volpicella et al., 2003). In order to verify the latter hypothesis we attempted to detect by phage display selection of a MTI-2 combinatorial library, mutants that could efficiently inhibit *HzTrypsin-S*. The library was produced by randomization of the P1P2P1P2 (APRIF) codons and was already successfully used to select mutant against bovine chymotrypsin (Ceci et al., 2003). The selected region lies in an external loop of the molecule, as deducible from the model of MTI-2 (Fig. 2).

The observation that selected mutants were inactive against both bovine trypsin and *HzTrypsin-S* (Table 2) prompted us to...
study, by a retromutagenesis approach, the relevance of single amino acids of mutated region in recovering activity. The mutant KNRLS obtained by the phage display approach, was used for restoring progressively the original P2 and P3 residues, proline and alanine respectively. We considered these only two changes on the basis that all the active MTI-2 mutants obtained by phage display against trypsin show a strict conservation of P2 and P3 residues (Ceci et al., 2003 and Table 1). Also Chy8, a MTI-2 derived chymotrypsin inhibitor obtained by phage display selection, shows conservation of these two positions (Ceci et al., 2003). Particularly CD analysis of the KNRLS mutant showed a reduction of helicity (Fig. 1) that could negatively affect the activity of the mutant, since the α-helix region is located near the reactive site (Zhao et al., 2002) as deduced from the MTI-2 structural model, obtained using the structure of A. thaliana trypsin inhibitor as template (PDB ID 1JXC) (Fig. 2).

The two retromutants KPRLS and APRLS are both active against trypsin (Table 2) and also show CD spectra similar to that of the wild-type (Fig. 1). Interestingly the one-step retromutant KPRLS is more active against trypsin than the two-step retromutant APRLS. Even if we cannot exclude a possible role of the two mutations in P′1 and P′2 in affecting the activity of the two mutants, this finding underlines the relevant role of proline in the P3 position, as observed for substrates of the serine protease thrombin (Harris et al., 2000). Zhao et al. (2002) compared the P3–P′3 loop conformation of the MTI-2 analogue ATTp with the same region of serine protease inhibitors from Bowman-Birk and squash families. By this analysis, the authors highlighted the particular role of the Pro residue in the P3 position, specific of ATTp, which causes a kink in the backbone of the reactive loop. Even if the P3 residue is not part of the MTI-2 α-helix region (residue 25–33), its changing dramatically affects the correct formation of the α-helix, as showed by CD analysis. This would have relevant conformational changes on the overall structure, leading to a non-functional reactive loop.

The small but detectable increase in negative ellipticity for the KPRLS mutant, in the absence of significant change in the CD profile, may arise from a greater chain rigidity of the mutant (Goodman et al., 1969; Litman and Schelmann, 1965). We have looked carefully at the homology model of KPRLS and found a close contact between the positively charged side-chain of Lys18 (P3) and the aromatic ring of Phe14 (supplementary material, Fig. S1). This type of cation–π interaction, with an optimal spacing between the two interacting amino acids (separated by three residues in a turn), was found to stabilize the polypeptide chain of model peptides (Ma and Dougherty, 1997; Shoemaker et al., 1990), and most likely help stabilize the reactive loop of MTI-2. The greater rigidity of the reactive loop arising from this interaction reflects in a greater negative ellipticity in the CD spectrum of the KPRLS mutant compared to wild-type and APRLS. Furthermore, the lysine in P3 position can partially offset the negative effect of P′1 and P′2 mutations on protein activity, thus explaining the 8-fold difference in the apparent Ki constants of KPRLS and APRLS (Table 2), the shorter and neutral alanine side-chain being unable to establish an analogous cation–π interaction with the phenylalanine ring in the latter mutant.

Unfortunately the recovery of the activity against bovine trypsin for the two mutants KPRLS and APRLS does not correspond to any activity of the two inhibitors against the insensitive HzTrypsin-S. This result leads to conclude that, at least for MTI-2, hardly the clue of protease insensitivity is in its interaction with the region of the active site of the enzyme, but probably resides in one, or more, structural features of the contact regions with the inhibitor. This conclusion is in accordance with results reported by Bayes et al. (2005) on the structural analysis of an insensitive (toward the potato carboxypeptidase inhibitor, PCI) carboxypeptidase from H. zeae and its comparison with a highly homologous sensitive carboxypeptidase from H. armigera. The analysis revealed that insensitivity can be explained on the basis of a different positioning of two of the main regions (the β8–α9 loop, and α7 together with the α7–α8 loop) that stabilize the carboxypeptidase–PCI complex, leading to a displacement of the active site entrance and improper interaction with the inhibitor.

The improper interaction of the reactive site with the protease active site, can also explain the larger number of MTI-2 mutants obtained in the case of phage display selection against HzTrypsin-S (19 different sequences out of the 19 sequenced...
phagemid molecules) than against bovine trypsin (only two different sequences out of the 14 sequenced molecules, with one of the two sequence detectable 13 times). Probably, a proper target enzyme, like bovine trypsin that is highly inhibited by MTI-2, can induce a strict selection of mutants, while the use of a relatively loosely binding enzyme, like the insensitive protease, can cause selection of a heterogeneous array of inactive mutants.

A further application of phage display strategy for the identification of mutants active against insensitive protease will only be possible after establishment of the specific interactions of MTI-2 with trypsins. The strict requirement for the Pro residue in P2 position of MTI-2 must also to be taken into account for future applications of the phage display approach.

Acknowledgements

We are grateful to Dr. Simone Scintilla for assistance in the recording of CD spectra. We also thank the University of Bari, the Consorzio Internuniversitario di Ricerca in Chimica dei Metalli nei Sistemi Biologici (CIRCMBS) and the Italian Inter-University Consortium for Biotechnologies (CIB) for support.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jplph.2010.05.025.

References


