Journal of Plant Physiology 167 (2010) 1507–1511

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.de/jplph

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

Mariateresa Volpicella[a,*], Claudia Leoni[a], Fabio Arnesano[b], Raffaele Gallerani[a], Luigi R. Ceci[c]

[a] Dipartimento di Biochimica e Biologia Molecolare, University of Bari "A. Moro", Via Amendola 165/A, 70126 Bari, Italy
[b] Dipartimento Farmaco-Chimico, University of Bari "A. Moro", Via Orabona 4, 70125 Bari, Italy
[c] Istituto di Biomembrane e Bioenergetica, C.N.R., Via Amendola, 165/A, 70126 Bari, Italy

ARTICLE INFO

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

ABSTRACT

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.

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 Z46816) 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 P_1 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

* Corresponding author. Tel.: +39 080 5443311; fax: +39 080 5443317.
E-mail address: m.volpicella@biologia.uniba.it (M. Volpicella).

0176-1617/$ – see front matter © 2010 Elsevier GmbH. All rights reserved.
doi:10.1016/j.jplph.2010.05.025

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

Xl-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.

MTI2-XhoI 5′-AAAAAAAAA CTC GAG AAA AGA GAG GCT GAA GCT GAT AGC GAG TGC CTG AAA GAA TAC-3′, restriction site XhoI.

MTI2-UGA 5′-GGGGGGAAT TCA CTG ATC AAA AGG GCT GTC GTT GCA GTA G-3′, restriction site EcoRI.

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

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

1P_F 5′-GC TTC CCT TTC TGC **AAG CCT CGG CTT CTG** CCG ACG-3′

1P_R 5′-CG TCG GCG AAA GCC GAG GCT TGC AGA AAG GGA AGC-3′

2AP_F 5′-GC TTC CCT TTC TGC **GCG CCT CGG CTT TCG** CCG ACG-3′

2AP_R 5′- CG TCG GCG AAA GCC GAG GCG CGC AGA AAG GGA AGC-3′

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 Z-Arg-Arg-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 immobilised 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 cm^2 dmol^{-1}) according to $[\theta] = 100 \cdot [\theta]\text{obs}/(C \cdot L \cdot N)$, where $[\theta]$obs 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 immunotubes (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 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 P_1 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 P_1 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.