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Biochemical characterization of a novel carboxypeptidase inhibitor from a variety of Andean potatoes

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ABSTRACT

Natural protease inhibitors of metallocarboxypeptidases are rarely reported. In this work, the cloning, expression and characterization of a proteinaceous inhibitor of the A/B-type metallocarboxypeptidases, naturally occurring in tubers of *Solanum tuberosum*, subsp. *andigenum* cv. Imilla morada, are described. The obtained cDNA encoded a polypeptide of 80 residues, which displayed the features of metallocarboxypeptidase inhibitor precursors from the Potato Carboxypeptidase Inhibitor (PCI) family. The mature polypeptide (39 residues) was named imaPCI and in comparison with the prototype molecule of the family (PCI from *S. tuberosum* subsp. *tuberosum*), its sequence showed one difference at its N-terminus and another three located at the secondary binding site, a region described to contribute to the stabilization of the complex inhibitor-target enzyme. In order to gain insights into the relevance of the secondary binding site in nature, a recombinant form of imaPCI (rimaPCI) having only differences at the secondary binding site with respect to recombinant PCI (rPCI) was cloned and expressed in *Escherichia coli*. The rimaPCI exhibited a molecular mass of 4234.8 Da by MALDI-TOF/MS. It displayed potent inhibitory activity towards A/B-type carboxypeptidases (with a *Ki* in the nanomolar range), albeit 2–4-fold lower inhibitory capacity compared to its counterpart rPCI. This result is in agreement with our bioinformatic analysis, which showed that the main interaction established between the secondary binding site of rPCI and the bovine carboxypeptidase A is likely lost in the case of rimaPCI. These observations reinforce the importance of the secondary binding site of PCI-family members on inhibitory effects towards A/B-type metallocarboxypeptidases. Furthermore, as a simple proof of concept of its applicability in biotechnology and biomedicine, the ability of rimaPCI to protect human epidermal growth factor from C-terminal cleavage and inactivation by carboxypeptidases A and B was demonstrated.

1. Introduction

Proteases (or peptidases) are enzymes that irreversibly cleave proteins by catalyzing peptide-bond hydrolysis. The human genome encodes about 560 proteases that represent around 2% of all human genes, indicating the importance of this class of enzymes (Quesada et al., 2009; Rawlings et al., 2012). Proteases play essential roles in multiple biological processes, and their hydrolytic activities need to be strictly controlled by several mechanisms including the action of specific inhibitors (Turk et al., 2012). One of the main groups of proteases is constituted of metallocarboxypeptidases (MCPs), which hydrolyze the peptide bond at the C-terminus of peptides and proteins by a zinc-dependent mechanism (Fernández et al., 2013; Jozic et al., 2002). MCPs were initially described as pancreatic proteases, but over the years they were found in other locations taking part in a huge variety of

Abbreviations: bCPA, carboxypeptidase A from bovine pancreas; hCPA1, human carboxypeptidase A1; hCPA2, human carboxypeptidase A2; pCPB, carboxypeptidase B from porcine pancreas; DMSO, dimethyl sulfoxide; EGF, epidermal growth factor; imaPCI, Imilia morada Andean Potato Carboxypeptidase Inhibitor; MALDI-TOF/MS, matrix-assisted laser desorption and ionization time-of-flight/mass spectrometry; MPCs, metallocarboxypeptidases; MPCI, metallocarboxypeptidase inhibitor; MS/MS, tandem mass spectrometry; PCI, Potato Carboxypeptidase Inhibitor; PIs, protease inhibitors; PPIs, proteinaceous protease inhibitors; rimaPCI, recombinant imaPCI; rPCI, recombinant PCI; RT-PCR, reverse transcription polymerase chain reaction.

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physiological processes such as blood coagulation/fibrinolysis, inflammation, prohormone and neuropeptide processing, local anaphylaxis, insect/plant-attack/defense strategies, and growth factors regulation, among others (Arolas et al., 2007; Fernández et al., 2013; Tanco et al., 2013). The dysregulated activity of some MCPs has been associated with several diseases in humans (Fernández et al., 2013); therefore, the use of protease inhibitors (PIs) has emerged as a promising tool in the development of therapeutic strategies for a large number of diseases. Many PIs with pharmacological uses are small molecules obtained by synthetic procedures (Abbenante and Fairlie, 2005; Fear et al., 2007; Fernández et al., 2013); however, natural PIs offer not only a greater chemical diversity and higher specificity, but also lower toxicity and hydrophobicity (Fear et al., 2007). Among natural PIs, the proteinaceous protease inhibitors (PPIs) are especially abundant in the storage organs of some families of the plant kingdom (*e.g.* Solanaceae). One of the most extensively studied PPIs in plants is the Potato Carboxypeptidase Inhibitor (PCI), isolated from tubers of *Solanum tuberosum* subsp. *tuberosum* L. cv. Desirée (Solanaceae), commonly known as the potato. The mature form of PCI is a single polypeptide chain of 39 amino acids stabilized by three disulfide bonds that determine a globular core of 27 residues. This particular conformation situates the PCI among the cystine-knot or T-knot superfamily proteins (Bateman and James, 2011; Bronsoms et al., 2003). From its globular core protrudes a C-terminal tail of five residues (residues 35-39) that is capable of inhibiting the catalytic activity of A/B-type MCPs by mimicking the substrate binding to their active site (Avilés et al., 1993; Marino-Buslje et al., 2000). Once the stable complex is formed, the C-terminal Gly of PCI is cleaved by the enzyme while the rest of the PCI tail remains tightly bound to the MCP's active site, thereby impeding further access of substrates (Arolas et al., 2004; Zhang et al., 2012). Besides the C-terminal tail that constitutes the primary binding site with the enzyme, a secondary surface including residues 15, 23 and 28–31 of PCI has also been proposed to contribute to the efficient interaction of the molecule with its target enzyme (Arolas et al., 2004; Marino-Buslje et al., 2000).

Reported herein is the identification of a novel member of the PCI-family present in *S. tuberosum* L. subsp. *andigenum* cv. Imilla morada, a variety of potato that grows along the Andean Cordillera in South America. The novel inhibitor, named imaPCI, was cloned starting from total RNA isolated from the tuber buds and expressed in *Escherichia coli* for its functional characterization. The recombinant product (rimaPCI) was identified and characterized using proteomic tools and the *K*i values of rimaPCI and recombinant PCI (rPCI) were determined against four selected A/B-type MCPs. Complementary to the kinetic assays, an *in silico* analysis was performed to further correlate the natural divergence in amino acids that constitute the secondary binding sites of imaPCI and PCI, with their different inhibitory properties. Finally, the ability of imaPCI to protect the epidermal growth factor from C-terminal cleavage and inactivation by MCPs was evaluated, as a simple proof of concept of its applicability in biotechnology and biomedicine. This work expands the current knowledge of PPIs present in potatoes, one of the most cultivated and consumed crops all over the world. Particularly, it paves the way for studying MCP-inhibitors (MCPIs) in Andean potatoes, which thanks to their thousands of varieties represent an extensive reservoir of molecules of huge diversity for biotechnological and pharmaceutical applications.

2. Results

2.1. Cloning and analysis of the cDNA sequence of the imaPCI precursor

In order to identify novel natural MCPIs, total RNA was isolated from tuber buds of the Andean potato Imilla morada variety and a cDNA of 360 bp was obtained by RT-PCR using primers based on conserved regions between the inhibitors of *S. tuberosum* subsp. *tuberosum* L. and *Solanum lycopersicum* L. (Solanaceae). The amplified cDNA was subjected to multiple sequence alignment using the BLAST program (Altschul et al., 1997), revealing a high degree of similarity with sequences of members belonging to the PCI family (inhibitor family I37 in MEROPS database (Rawlings et al., 2014). Particularly, the obtained sequence showed 97% identity (Score value = 603; *E*-value = 6e−169) with *S. tuberosum* metallocarboxypeptidase inhibitor IIa (PCI) precursor mRNA (GenBank: NM_001288119.1). The protein product encoded by 243 bp of the obtained cDNA was named imaPCI precursor and uploaded to the GenBank database under accession number KM359974. The nucleotide and deduced amino acid sequences of the imaPCI precursor are shown in Fig. 1A. The sequence of imaPCI precursor was compared with the amino acid sequence of the PCI precursor (GenPept: NP_001275048.1), in order to identify characteristic regions and residues. The typical organization for the PCI family members (González et al., 2003) was also observed for the imaPCI precursor. That is, a putative signal peptide (only partially amplified through the cloning strategy used in this work), an N-terminal pro-segment of 27 amino acids followed by 39 residues of the mature imaPCI protein, and a 7 residue-extension at the C-terminus (Fig. 1A). Although imaPCI and PCI precursors share 97% nucleotide sequence identity, the mature domains of these inhibitors differ in 4 of the 39 residues (11%) of their primary sequences. As shown in Fig. 1B, most of the non-conserved residues between both sequences (i.e. positions 3, 23, 29 and 30 of the mature protein numbering) are located in positions corresponding to the region known as secondary binding site of these inhibitors (i.e. positions 15, 23 and 28–31), whereas only one amino acid along the rest of the sequences is different, i.e. His3 in PCI and Asp3 in imaPCI. The six conserved cysteine residues, described to be involved in disulfide bond formation (Cys8–Cys24; Cys12–Cys27 and Cys18–Cys34, mature protein numeration) (Chang et al., 1994), are also present in the imaPCI sequence. Moreover, when the amino acid sequence of the mature imaPCI was analyzed by the KNOTER1D tool of the KNOTTIN database software (Gracy et al., 2008), the expected knottin structure was predicted with a knoter1d score of 41 (knottin predictive score > 21). No N-glycosylation or phosphorylation putative sites along the mature imaPCI sequence were predicted by NetNGlyc 1.0 Server (Blom et al., 2004) and NetPhos 2.0 Server (Blom et al., 1999), respectively. An average molecular mass of 4214.7 Da and a pI value of 5.4 were calculated for the mature inhibitor using the Compute pI/Mw tool (Gasteiger et al., 2005). In brief, the imaPCI amino acid sequence shares features described for the members of the MEROPS inhibitor family I37.

2.2. Heterologous expression and purification of rimaPCI

The differences in the secondary binding site are especially interesting since studies based on site-directed mutagenesis and alanine-scanning mutagenesis of recombinant PCI (rPCI), have shown that the secondary binding site of PCI significantly contributes to the stabilization of its inhibitory complex with bCPA (Arolas et al., 2004; Marino-Buslje et al., 2000). Consequently, imaPCI constitutes a natural variant of PCI useful to investigate the importance of the secondary binding site in carboxypeptidase inhibition. For this reason, and for simplicity of the analysis, an imaPCI variant (rimaPCI) was recombinantly produced that maintains the divergences in the secondary binding site, but lacks the differences in its N-terminus with respect to the rPCI sequence (Fig. 2). Interestingly, the N-terminus of rPCI (and therefore also of rimaPCI) corresponds to the reported sequence of isoinhibitor IIa isolated from potato (Hass and Derr, 1979), i.e. a Glu residue