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Biochemical characterization of the YBPCI miniprotein, the first carboxypeptidase inhibitor isolated from Yellow Bell Pepper (*Capsicum annuum* L). A novel contribution to the knowledge of miniproteins stability

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ABSTRACT

The cystine-knot metallocarboxypeptidase inhibitors (MCPIs) are peptides that contribute to control proteolytic activity, involved in storage, growth and maintenance of plants. Lately studies reported several MCPIs with potential use in biomedical applications; as anti-cancer, anti-thrombotic, anti-malaric and anti-angiogenic agents. We report the isolation, purification, chemical stability and biochemical characterization of a novel carboxypeptidase A inhibitor (YBPCI) isolated from *Capsicum annuum* L. var. Yellow Bell Pepper, the first cystine-knot miniprotein (CKM) of the species. We demonstrate the stability of YBPCI (IC50 = 0.90 μg/ml) to high temperatures, high salt concentration and extreme pH values. MALDI-TOF/MS analysis detected a molecular weight of 4057 Da, and peptide mass fingerprint resulted in no matches with other protease inhibitors. *In vitro* gastrointestinal digestion subjecting YBPCI to pH 2 incubation and proteolytic attack resulted in complete inhibitory activity. To summarize, there are no reports to date of carboxypeptidase inhibitors in *C. annuum* species, giving our report much more relevance.

1. Introduction

Carboxypeptidases (CPs) are enzymes that cleave proteins from the C-terminus, usually one residue at a time, playing an essential role in degradation, processing, and modulation of proteins and peptides [1]. CPs were initially considered as degrading enzymes associated with protein catabolism, but over the years evidence demonstrates that some CPs play key roles in controlling a vast variety of biological processes [2]. Among CPs, metallocarboxypeptidases (MCPs) are exopeptidases that hydrolyze the C-terminal amide bond by a zinc-dependent mechanism [3]. In humans, the dysregulated activity of some MCPs has been associated with several diseases such as blood coagulation/fibrinolysis, inflammation, carcinogenesis, epilepsy and febrile seizures, among others [1,2,4,5]. According to these studies, the use of protease inhibitors (PIs) in order to regulate MCPs action has emerged as a potential tool for the development of therapeutic strategies for a large number of diseases; as anticarcinogenic, anti-angiogenic and anti-thrombotic, among others [6–9]. Furthermore, although protease inhibitors have been considered only as antinutritional factors, they have regained interest in recent years because of their potential for drug development and positive dietary effects.

The application of natural PIs offers great advantages over those of synthetic origin due to their lower toxicity and hydrophobicity [10]; however, naturally occurring metallocarboxypeptidase inhibitors

Abbreviations: CKM, cystine-knot miniproteins; MCPIs, metallocarboxypeptidase inhibitors; YBPCI, Yellow Bell Pepper Carboxypeptidase Inhibitor; MALDI-TOF/MS, matrix-assisted laser desorption and ionisation time-of-flight/mass spectrometry; PMF, peptide mass fingerprint; CPs, carboxypeptidases; MCPs, metallocarboxypeptidases; PIs, protease inhibitors; PCI, Potato Carboxypeptidase Inhibitor; ACI, *Ascaris suum* Carboxypeptidase Inhibitor; LCI, *Hirudo medicinalis* Carboxypeptidase Inhibitor; TCI, Ticks Carboxypeptidase Inhibitor; H1TCI, *Hemaphysalis longicornis* Carboxypeptidase Inhibitor; NvCI, *Nerita versicolor* Carboxypeptidase Inhibitor; ECI, Endogenous Carboxypeptidase Inhibitor; SmCI, *Sabellastarte magnifica* Carboxypeptidase Inhibitor; EGF, Epidermal Growth Factor; PSI, Paprika Seeds Inhibitor; CaTI, *Capsicum annuum* Trypsin Inhibitor; PIJP, Protease Inhibitor from Jalapeño Pepper; CPA, Carboxypeptidase A; SDS, sodium dodecyl sulphate; βME, β-mercaptoethanol; TEMED, *N,N,N′,N′*-tetramethyl ethylene diamine; BSA, bovine serum albumin; CPAU, Carboxypeptidase A unit

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(MCPIs) have only been identified in: *Solanaceae* family, such as potato (PCI) and its close homologue in tomato [11,12]; the intestinal parasite *Ascaris suum* (ACI) [13]; the medicinal leech *Hirudo medicinalis* (LCI) [14]; the tick *Rhipicephalus bursa* (TCI) [15] and *Hemaphysalis longicornis* (H1TCI) [16]; the marine mollusk *Nerita versicolor* (NvCI) [17]; in rat and human tissues (latexin or endogenous carboxypeptidase inhibitor (ECI)) [18] and in the marine ringworm *Sabellastarte magnifica* (SmCI) [19]. Moreover, the well-studied PCI had shown multiple bioactive functions: antitumor properties as an Epidermal Growth Factor (EGF) antagonist [6], antimalaric agent by the growth inhibition of the parasite Plasmodium falciparum [20], antithrombotic agent [7], and *in vitro* antifungal properties against phytopathogens were reported [21]. All of these MCPIs belong to the cystine knot or T-knot family, and share similar features such as their small size (39-75 residues) and stabilization by several disulphide bridges; typically comprising six Cys residues with conserved disulphide bonds. Despite the common knottin motif, knottin-type peptides have hypervariable sequences, differing by their amino acid sequences, the length between disulphide bonds, and the linear and cyclic nature of the peptide backbone [22]. Plant knottin-type peptides, particularly the subfamily of cyclotides, have been reported to possess high thermal, chemical, and enzymatic stability [23]. Cyclotides are also resistant to gastrointestinal proteases like trypsin, chymotrypsin, pepsin, or elastase, and certain members of cyclotide family can even penetrate the intestinal mucosa excised from rats [24].

The genus *Capsicum* (vegetable pepper) was initially domesticated in Mexico, northern Central America and the Andean region of South America. By the end of the 20th century, Argentina was the main producer in South America of vegetable pepper with a cultivation of about nine thousand hectares of pepper per year, representing an approximate production of 300,000 tons. *Capsicum* varieties have different uses as food products, either as fresh or processed vegetables, spices or even medicines and pest control, which makes this crop of immense cultural and economic importance [25]. *Capsicum annuum* L. presents a large number of varieties depending on the characteristics of the fruit (elongated pepper, four hulls, chili vinegar, yellow pepper, orange, violet, among others) of which a few have been studied so far. Reports of protease inhibitors from *C. annuum* are limited to the serine proteinase type. PSI-1.1 and PSI-1.2 were isolated from paprika seeds [26], CaTI was isolated from chilli pepper seeds (UENF 1381) [27], CapA1 and CapA2 were isolated from *C. annum* var. Phule Jyoti leaves [28] and finally PIJP was isolated from jalapeño pepper [29], all of them presented inhibitory activity against trypsin and chymotrypsin. In this work, we isolated and characterized a carboxypeptidase inhibitor from *C. annuum* L. var. Yellow Bell Pepper named YBPCI. To our best knowledge, this is the first report of a metalloproteinase inhibitor isolated from *C. annuum*. Furthermore, we demonstrate that YBPCI displays resistance to *in vitro* gastrointestinal digestion, suggesting that PIs could reach the colon in intact form. These results encourage the study on possible pharmacological applications that may be present through the ingestion of these inhibitors, either for their potential antiparasitic or antibacterial action on pathogenic bacteria.

2. Material and methods

2.1. Materials

Carboxypeptidase A (CPA) from bovine pancreas, Pepsin from porcine gastric mucosa, Pancreatin from porcine pancreas, sodium chloride, tris (hydroxymethyl) aminomethane, sodium dodecyl sulphate (SDS), β-mercaptoethanol (βME), Coomassie Blue G-250, *N,N,N′,N′*-tetramethyl ethylene diamine (TEMED) and bovine serum albumin were supplied by Sigma-Aldrich (U.S.A.). N-(4-methoxyphenylazoformyl)-phenylalanine-OH potassium salt was obtained from Bachem (Switzerland).

2.2. Crude extract preparation

C. *annuum* seeds were collected from nearby areas around La Plata (Argentina) and washed thoroughly in distilled water. The dry seeds (30 g) were ground using a blender with addition of 3 vol of 0.01 M phosphate buffer, 0.1 M NaCl, pH 7.4. The mixture was incubated 3 h at 4 °C and filtered with gauze. The suspension was centrifuged at 7000 g for 30 min at 4 °C, and the supernatant was collected and frozen at -20 °C until analysis.

2.3. Carboxypeptidase a inhibition activity

The CPA activity assay is based on the hydrolysis of the substrate N-(4-methoxyphenylazoformyl)-Phe-OH [30]. For the inhibition experiment a fixed concentration of CPA solution (50 nM) was pre-incubated with different concentrations of inhibitor in 0.1 M Tris-HCl buffer (pH 7.4) containing 0.2 M NaCl. After 15 min at 37 °C, the chromogenic substrate was added to the reaction mix at a concentration of 1 mM and, immediately, the absorbance at 340 nm was measured every minute for a minimum of 15 min. The protease inhibitor activity was expressed in terms of percent inhibition of CPA activity throughout the course of study, by using the comparison of absorbance changes of the test and the control, according to the equation:

$$CPA\ inhibition\ (\%) = \left(1 - \frac{[\Delta Abs(340nm)/\Delta t(min)]_{Inhibitor}}{[\Delta Abs(340nm)/\Delta t(min)]_{Control}}\right) x\ 100$$

Inhibitory activity (IA) was defined according to Tellechea et al. (2016) [31]. Measurements were carried out in triplicate.

2.4. Protein estimation

Protein concentration was measured by the Bradford's method using bovine serum albumin (BSA) as standard (0-1 mg/ml). 20 µl of sample and standard were mixed with 200 µl of reagent for 10 min at room temperature. Afterwards, protein concentration was measured by absorbance at 595 nm (Tecan Infinite M200 PRO).

2.5. Purification

2.5.1. Partial purification by heat treatment

1 ml of crude extract (400 µg/ml) was subjected to 60 °C, 70 °C, 80 °C, 90 °C or 100 °C for 5 min, thermally denatured proteins were removed by centrifugation (10000 g, 40 min, 4 °C). 100 µl of each supernatant -including a crude extract control- was employed as sample in the CPA inhibitory activity assay and residual activity was determined as described previously.

2.5.2. Affinity chromatography purification

A sample of heat treated extract containing 200 µg/ml of protein was loaded to a carboxypeptidase A-glyoxyl-agarose column (1.5 × 10 cm) [32] previously equilibrated with 0.1 M Tris-HCl buffer (pH 8.0) containing 0.2 M NaCl. In the first step unbound proteins were eluted with equilibration buffer, and then affiliated proteins were eluted with HCl pH 2.0. The eluent was adjusted to pH 7.0 and the fractions exhibiting inhibitory activity were pooled.

2.6. Characterization of protease inhibitor

2.6.1. SDS-tricine-PAGE

SDS-Tricine-PAGE was used to determine the purification advance of the protease inhibitor during purification. Samples were mixed with sample buffer (Tris 0.13 M, SDS 2%, β-mercaptoethanol 5% v/v, glycerol 8% v/v, bromophenol blue 0.002% p/v, pH 6.8) and incubated 5 min at 100 °C, then subjected to denaturing electrophoresis at a constant current of 15 mA per gel using a Mini – Protean III dual slab