Protein Expression and Purification xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

Purification and characterisation of an antifungal protein, MCha-Pr, from the intercellular fluid of bitter gourd (*Momordica charantia*) leaves

Beibei Zhang [1,2], Chengjian Xie [2], Yunming Wei, Jing Li, Xingyong Yang *

The Chongqing Key Laboratory of Molecular Biology of Plant Environmental Adaptations, Chongqing Normal University, 401331 Chongqing, China
The College of Life Science, Chongqing Normal University, 401331 Chongqing, China

ARTICLE INFO

Article history:
Received 13 July 2014
and in revised form 11 September 2014
Available online xxxx

Keywords:
Bitter gourd
Intercellular fluid
Antifungal protein
Isolation

ABSTRACT

An antifungal protein, designated MCha-Pr, was isolated from the intercellular fluid of bitter gourd (*Momordica charantia*) leaves during a screen for potent antimicrobial proteins from plants. The isolation procedure involved a combination of extraction, ammonium sulphate precipitation, gel filtration on Bio-Gel P-6, ion exchange chromatography on CM-Sephadex, an additional gel filtration on HiLoad 16/60 Superdex 30, and finally, HPLC on a SOURCE 5RPC column. Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry indicated that the protein had a molecular mass of 25733.46 Da. Automated Edman degradation was used to determine the N-terminal sequence of MCha-Pr, and the amino acid sequence was identified as V-E-Y-T-I-T-G-N-A-G-N-T-P-G-G. The MCha-Pr protein has some similarity to the pathogenesis-related proteins from *Atropa belladonna* (deadly nightshade), *Solanum tuberosum* (potato), *Ricinus communis* (castor bean), and *Nicotiana tabacum* (tobacco). Analysis of the circular dichroism spectra indicated that MCha-Pr predominantly contains α-helix and β-sheet structures. MCha-Pr had inhibitory effects towards a variety of fungal species and the 50% inhibition of fungal growth (IC_{50}) for *Alternaria brassicae*, *Cercospora personata*, *Fusarium oxysporum*, *Mucor* sp., and *Rhizoctonia solani* are 33 μM, 42 μM, 37 μM, 40 μM, and 48 μM, respectively. In addition, this antifungal protein can inhibit the germination of *A. brassicae* spores at 12.5 μM. These results suggest that MCha-Pr in bitter gourd leaves plays a protective role against phytopathogens and has a wide antimicrobial spectrum.

Introduction

Disease development in plants is a sporadic event even though plants are constantly challenged by a great diversity of pathogenic organisms at each stage of their lifecycle. Plants synthetise a variety of proteins/peptides to defend themselves from parasites and pathogens, such as bacteria, fungi, and viruses. Since plants are able to protect themselves from these pathogens, serious economic losses are often diminished [1,2]. These protective proteins and peptides are structurally diverse and are comprised of allergen-like peptides [3], chitinases [4], cyclophilin-like proteins [5], defensins and defensin-like proteins [6,7], glucanases [8], lipid transfer protein-like proteins [3,8], peroxidases [9,10], protease inhibitors [11], ribosome-inactivating proteins [12,13], thaumatin-like proteins [14], and other proteins and peptides with antimicrobial activity [15–17]. Seeing that antimicrobial proteins are important antibiotic resistance resources and transgenic expression of these proteins or peptides has been studied as a means to increase crop disease resistance [18], much effort has been devoted to uncovering novel, antimicrobial, plant-produced peptides and proteins that may be potential candidates for use in agricultural, medicinal, and food safety [1,2,17].

Momordica charantia L. (Cucurbitaceae), also known as bitter gourd, bitter melon, or balsam pear, is a widely cultivated vegetable and medicinal herb in many Asian countries and has been shown to have health benefits, particularly hypoglycemic effects and anti-diabetic properties towards mankind and animals [19,20]. Bitter gourd is also used as a carminative, an emmenagogue, for the treatment of colic, and also as an antiviral, anthelmintic, antimalarial, and antimicrobial remedy [19,21–23]. In this study, we purified an antifungal protein from the intercellular fluid of bitter gourd leaves with homology to some plant pathogenesis-related (PR) proteins. We used three different chromatographic methods for this purification. Further studies revealed that the protein inhibits the growth of some fungi.

* Corresponding author at: The College of Life Science, Chongqing Normal University, Huxi, Shapingba District, Chongqing 401331, China. Tel./fax: +86 23 65910315.
E-mail address: yangxy94@swu.edu.cn (X. Yang).
[1] Current address: Sichuan Academy of Food and Fermentation Industries, China.
[2] These authors contributed equally to the study.

http://dx.doi.org/10.1016/j.pep.2014.09.008
1046-5928/

Materials and methods

Materials

Bitter gourd (*M. charantia* L.) seeds were purchased from a local market (Chongqing, China) and sprouted in a field. The fungi, *Aspergillus niger*, *Alternaria brassicae*, *Cercospora personata*, *Fusarium oxysporum*, *Mucor* sp., *Rhizoctonia solani*, and *Verticillium dahliae*, were kindly provided by the Department of Plant Pathology, Southwest University (Chongqing, China). Bio-Gel P-6 was purchased from Bio-Rad (CA, USA). HiPrep 16/10 CM FF, HiLoad 16/60 Superdex 30 and SOURCE 5RPC columns were purchased from GE Healthcare (USA). Acetonitrile was purchased from Fisher Scientific Inc. (NJ, USA).

Soluble intercellular protein (SIP) preparation

The SIP was recovered by an infiltration-centrifugation method according to the method of Delannoy et al. [24] with minor modifications. Briefly, fresh bitter gourd leaves (1540 g) were cut from eight-week-old plants and soaked in cold 0.05 M sodium acetate buffer (buffer SA), pH 5.0 for 8 min. A vacuum was then applied (0.08 MPa) to the leaves for 10 min. After release of the vacuum, the leaves were surface-dried with bibulous paper, wrapped in a Miracloth filter and hung in a centrifuge tube (Sorvall, GSA). After centrifugation at 6500×*g* for 10 min at 4 °C, the solution in the centrifuge tubes was recovered. The SIP extract was centrifuged for 25 min at 16,500×*g* at 4 °C. The supernatant (1260 mL) was precipitated with solid ammonium sulphate (541.8 g) at 65% saturation for 4 h at 4 °C. The pellet was obtained by centrifugation at 16,500×*g* for 15 min and was then dissolved in 50 mL 0.05 M buffer SA (pH 5.0).

Isolation of the antifungal protein

The SIP solution was passed through a Bio-Gel P-6 (16 mm × 300 mm) gel filtration column on an ÄKTA Prime system (GE Healthcare, USA) to remove the ammonium sulphate. The column was first equilibrated with 20 mM buffer SA (pH 5.2). The protein was then eluted using the same buffer at a flow rate of 1.0 mL min^{-1}. The antifungal activity against *A. niger* was monitored in each fraction. The antifungal fraction was used for a cation exchange chromatography step on a HiPrep 16/10 CM FF column pre-equilibrated with 20 mM buffer SA (pH 5.2) on an ÄKTA Prime system. Following removal of the unabsorbed proteins with the above buffer, the absorbed proteins were eluted with a linear gradient of NaCl (0–1 M) in the same buffer. The antifungal fractions were pooled, concentrated and centrifuged at 16,500×*g* for 10 min, and the supernatant was then applied to another gel filtration column, a HiLoad 16/60 Superdex 30 column, that was pre-equilibrated with the same buffer on the same chromatography system. The column was eluted with 20 mM buffer SA (pH 5.2) at a flow rate of 0.8 mL min^{-1}. After the antifungal fraction was concentrated and centrifuged at 16,500×*g* for 10 min, the supernatant was further purified by reversed-phase high-performance liquid chromatography (HPLC) using a SOURCE 5RPC column (4.6 mm × 150 mm, 5 μm) equilibrated with distilled water on an ÄKTA Explorer 10S system (GE Healthcare, USA). The fraction was eluted with distilled water over 5 min followed by a linear gradient of 0–50% acetonitrile over 50 min at a flow rate of 1 mL min^{-1}. The individual peak fractions were collected and then concentrated in 2-kDa molecular weight cut-off dialysis tubing (Sigma, USA) with 20 kDa molecular mass of polyethylene glycol as water absorber. After dialysis against ultra purified water, the samples were further analysed. All purification steps were performed at room temperature, and the column effluent was monitored by absorbance at 280 nm.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was performed according to the method described by Laemmli [25]. The proteins were separated in an electrophoresis cell (Bio-Rad, CA, USA), and low molecular weight markers from Bio-Rad (USA) were used. The slab gel was one-mm-thick gel and was comprised of a 15% polyacrylamide separating gel with a 4% stacking gel. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R250 (Amersham Biosciences, Sweden). The molecular weight of the purified antifungal protein was determined by comparison of its electrophoretic mobility with that of the molecular weight marker proteins (Amersham Biosciences, Sweden). The molecular weight markers are comprised of β-galactosidase (115.5 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (37 kDa), soybean trypsin inhibitor (29 kDa), lysozyme (19.7 kDa), and aprotinin (6.7 kDa).

Protein determination and N-terminal amino acid sequence analysis

The protein concentration was determined using the method of Bradford with bovine serum albumin as the standard [26]. All protein assays were performed in triplicate. To determine the amino acid sequence of MCha-Pr, the purified sample was subjected to SDS–PAGE as described above and then electroblotted to a polyvinylidene difluoride membrane (Bio-Rad, CA, USA). The protein bands were visualised with Coomassie Brilliant Blue R-250 and then excised from the membrane. The N-terminal amino acid sequence determination was performed by the Shanghai Sangon Biological Engineering Technology & Services Co. Ltd. (Shanghai, China) on a protein sequencer (model No. 491, Applied Biosystems, USA).

Mass spectrometry and circular dichroism (CD) spectroscopy

The molecular mass of the MCha-Pr was determined by matrix assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS) using a REFLEX III mass spectrometer (Bruker Daltonics). MCha-Pr was mixed with an equal volume of matrix and 2 μL of this mixture was applied onto a MALDI sample plate and allowed to crystallise at room temperature.

CD measurements were performed using a Jasco J-810 spectropolarimeter over a wavelength range of 190–240 nm under constant N_2 purging according to the manufacturer's instructions (Jasco). A scan speed of 50 nm min^{-1}, a time response of 1 s, and a bandwidth of 1 nm were used. The measurements were performed on MCha-Pr samples at a protein concentration of 0.08 mg mL^{-1} in deionised water in quartz cuvettes with 1 mm path length. The spectra were typically recorded as an average of 16 scans.

Antifungal activity assay

The fungi, *A. brassicae*, *C. personata*, *F. oxysporum*, *Mucor* sp., *R. solani* and *V. dahliae* were utilised for the antifungal activity assay. The in vitro antifungal activity assay was performed as described by Yang et al. [11] with minor modifications. Briefly, the assay was performed in 60-mm Petri dishes containing 10 mL potato dextrose agar at one-third strength (7% potato, 0.7% dextrose, and 2% agar, natural pH). The mycelia were initially grown on the plates at 28 °C to obtain colonies ranging in size from 30 to 40 mm in diameter. The potential antifungal compounds that were dissolved in ultra purified water were then loaded into a hole in

Please cite this article in press as: B. Zhang et al., Purification and characterisation of an antifungal protein, MCha-Pr, from the intercellular fluid of bitter gourd (*Momordica charantia*) leaves, Protein Expr. Purif. (2014), http://dx.doi.org/10.1016/j.pep.2014.09.008