Peptides 32 (2011) 1151–1158

Contents lists available at ScienceDirect

Peptides

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

An antifungal peptide from *Fagopyrum tataricum* seeds

Jing-Jun Ruan, Hui Chen*, Ji-Rong Shao, Qi Wu, Xue-Yi Han

College of Life Science, Sichuan Agriculture University, Yaan 625014, Sichuan, People's Republic of China

ARTICLE INFO

Article history:
Received 25 January 2011
Received in revised form 16 March 2011
Accepted 16 March 2011
Available online 29 March 2011

Keywords:
Fagopyrum tartaricum
Trypsin inhibitor
Phytopathogenic fungi
Purification

ABSTRACT

A major trypsin inhibitor was isolated and characterized from the seeds of the tartary buckwheat (*Fagopyrum tataricum*) (FtTI) by ammonium sulfate precipitation, ion exchange chromatography and centrifugal ultrafiltration. SDS-PAGE analysis under reducing condition showed that FtTI is a single polypeptide chain with a molecular mass of approximately 14 kDa. The complete amino acid sequence of FtTI was established by automatic Edman degradation and mass spectrometry. It was found that the trypsin inhibitor molecule consists of 86 amino acid residues containing two disulfide bonds which connect Cys^{8} to Cys^{65} and Cys^{49} to Cys^{58}. The active site of the inhibitor was found to contain an Asp^{66}–Arg^{67} bond. MALDI-TOF analysis showed that FtTI has two isoforms (Mr: 11.487 and 13.838 kDa). Dixon plots revealed a competitive inhibition of trypsin with inhibition constants (Ki) of 1.6 nM. Analysis of the amino acid sequence suggests that FtTI is a member of the protease inhibitor I family. What is more, FtTI exhibited strong inhibitory activity against phytopathogenic fungi.

1. Introduction

Tartary buckwheat (*Fagopyrum tataricum*) is an ancient dicotyledonous crop belonging to Polygonaceae family. It is cultivated under low input conditions and adapted to marginal lands with harsh environments. Besides its benefits for human consumption due to the high quality proteins with a well balanced amount of essential amino acids in the seeds [20], tartary buckwheat is also important as a pharmaceutical plant. It can reduce the blood cholesterol and regulate the blood sugar, blood lipid and blood pressure in human body [42]. Due to harsh environments and evolution, plants can adapt a number of efficient mechanisms providing for their protection against phytopathogens and insect pests. Among these mechanisms the use of different plant proteins exhibiting toxic or antimetabolic activities toward pathogens are of great interest. These proteins can be to some extent involved in the creation of a protective barrier at early stages of infection. Proteinase inhibitors (PIs) can also be considered as protective proteins because they inhibit the activity of proteolytic enzymes of both insect pests and pathogenic microorganisms that attempt to penetrate the host plant tissues.

Plants counteract this by synthesis of constitutive and induced proteinase inhibitors. Most previously studied inhibitors found within seeds are inactive toward their own proteinases, but can efficiently inhibit proteolytic enzymes of animal and bacterial origin as well as proteinases of the alimentary canal of insect pests [12]. Although the biological role of protein proteinase inhibitors is not still sufficiently clear, it has been suggested that they may perform five main functions: as endogenous insecticides, by inhibiting the proteinases inoculated by pest insects and pathogenic microbes; in plant defenses against abiotic or mechanical stressors; as regulators of proteinases, to prevent premature degradation of storage proteins during their synthesis, processing, and packaging into protein bodies during seed development; as storage proteins, containing reduced sulfur; and in protection of the embryo sac, allowing proper endosperm and embryo formation [21]. However, plant protease inhibitors continue to attract the attention of researchers because of their increasing use in medicine. In recent years, it has been found that proteinase inhibitors can induce apoptosis of cancer cells in vitro. Furthermore, it has been reported that the Bowman-Birk family of inhibitors, obtained from soybeans and other legumes, are potentially nutritionally relevant anti-carcinogens, particularly with respect to colon cancer [41]. Therefore, PIs have been receiving attention as potential anticancer agents [15]. Protein serine PIs are widely distributed in plants, especially in seeds. The presence of PIs in seeds is important in that they interfere with both human and livestock protein digestion and absorption because of their ability to inhibit animal digestive enzymes and hence the utilization of dietary protein [19,26]. Most of these inhibitors bind to cognate enzymes according to a common substrate-like canonical mechanism forming stable complexes with target proteinases, and blocking, altering or abolishing access to the enzyme active site [5,41]. The stability of proteinase–PI complex is largely brought about by intramolecular

* Corresponding author. Tel.: +86 0835 2886134; fax: +86 0835 2886136.
E-mail address: chen62hui@yahoo.com.cn (H. Chen).

0196-9781/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.peptides.2011.03.015

interactions with disulfide bond, hydrogen bond, and hydrophobic interaction, involving the primary binding loop (reactive site loop) structure [6,24]. Most serine PIs is low-molecular mass molecules (3–25 kDa) that inhibit trypsin and/or chymotrypsin. Based on the homologies evident in their primary structures, localization of the reactive sites, disulfide bridge topology, mechanism of action, three-dimensional structure, and stability to heat and denaturing agent, the plant inhibitors of serine proteinases have been grouped into seven distinct families: Kunitz, Bowman-Birk, Potato I, Potato II, Squash, Cereal and Mustard [4,9]. Among the serine PIs, the Kunitz trypsin inhibitor superfamily has generated much prominence for its specific activity against trypsin-like serine proteinases, with no inhibition of other proteinase classes [6,16]. Trypsin inhibitors are PIs that block protein breakdown by trypsin. Plants have developed both physical and molecular strategies to limit consumption by insect pests while attracting insect pollinators. Potato types I and II serine protease inhibitors are produced by plants as a defense mechanism against these insects and microbes [17]. Potato type I and II inhibitors can target the digestive serine proteinases trypsin and chymotrypsin, the major enzymes contributing to protein digestion in the gut of insect [1].

Several PIs from common buckwheat seeds have been reported [2,3,33,34,43], however, the PIs of tartary buckwheat are still unknown. The present work has been carried out to unveil the presence of serine-protease inhibitors also in tartary buckwheat seeds. The paper describes the purification, structural characterization, biochemical properties and antifungal activity of a trypsin inhibitor FtTI, which represents the first potato type I protein isolated from tartary buckwheat seeds.

2. Materials and methods

2.1. Materials

Tartary buckwheat seeds were manually harvested from Liang Mountain, in the Liang Shan region of Sichuan province, China. Seeds were air-dried and stored at −20 °C before use.

2.2. Isolation of trypsin inhibitor

Dry seeds of tartary buckwheat (*F. tataricum Gaertn.*) were ground in an electric mill and the fine powder was extracted with buffer A (67 mM K, Na phosphate, pH 6.8) at 4 °C for 24 h. The suspension was centrifuged at 14,000 × *g* for 15 min at 4 °C, then dry $(NH_4)_2SO_4$ was added to the supernatant (to 80% saturation) [2]. The precipitate generated was allowed to stand for 12 h at 4 °C, then was centrifuged at 14,000 × *g* for 15 min at 4 °C and dialyzed for 24 h at 4 °C against buffer A. The total preparation of proteins (10 mg) was applied to an anion-exchange DEAE52-Cellulose column (1.5 × 20 cm) in buffer B (10 mM K, Na phosphate, pH 6.8), and the protein was subsequently eluted with a linear gradient of 0–250 mM NaCl at a flow rate of 0.75 mL/min. The active fractions were pooled, dialyzed against buffer B and lyophilized. Lyophilized fractions were diluted using buffer A and subjected to affinity chromatography on a trypsin-Sepharose 4B column (1.5 × 20 cm). The inhibitors of buffer A were adsorbed on the affinity column which contained 0.5 M potassium chloride at 4 °C for 4 h. Unbound proteins were thoroughly washed off and inhibitors were eluted with 1 mM HCl, pH 2.7, containing 0.5 M KCl. The fractions with TI activity were pooled and dialyzed against buffer B. The dialysis procedure was carried out in benzoylated dialysis tubing (Sigma, St. Louis, USA) with an exclusion limit of 8000 Da. This fraction was applied to ultrafiltration equipment (Minipore co., USA) to concentrate for further use.

2.3. Trypsin inhibition assay and Ki determination

The inhibitory activity on bovine pancreatic trypsin was determined by measuring the hydrolytic activity toward the substrate BAPNA [39]. The different concentrations of inhibitor were incubated with 5.6×10^{-7} M trypsin (final concentration in 1.5 mL assay volume) at 30 °C for 15 min in 50 mM Tris–HCl buffer, pH 8.0 containing 20 mM $CaCl_2$ and 0.001 N HCl. After 15 min incubation, 1.0 mL of 0.5 mM BAPNA was added. After incubation of another 10 min at 30 °C, the reactions were stopped by adding 200 μL of 10% acetic acid. The changes in absorbance at 410 nm were recorded at 30 °C against a blank solution containing 1.5 mL of the substrate solution in same buffer with a SHIMADZU Uvmini-1240 spectrophotometer. The amount of substrate (BAPNA) hydrolysis by the enzyme was calculated using a molar extinction coefficient of 8800 M^{-1} cm^{-1} at 410 nm. One unit of the inhibitor activity produced a decrease in absorption of the solution by 0.1 units in the trypsin activity assay.

Kinetic studies over a range of FtTI concentrations were performed to determine the inhibition constant (Ki) from a Dixon plot using BAPNA as a substrate [10,32,38]. Studies were performed by adding a range of inhibitor concentrations to a fixed amount of trypsin (final concentration was 0.32 nM) at two different substrate concentrations of 1.0 and 5.0 mM. The final inhibitor concentrations used were 0, 1.6, 3.3 and 5.0 nM. The reciprocal velocity ($1/v$) versus inhibitor concentrations [I], for each substrate concentration, [S1] and [S2], were plotted (Dixon plots). A single regression line for each [S] was obtained, and the Ki was calculated from the intersection of the two lines. Each measurement was made in triplicate and differences were <5%.

2.4. Protein estimation

Protein contents were estimated by Coomassie blue staining (dye-binding method) [7] and from the absorbance of the SHIMADZU Uvmini-1240 spectrophotometer at 280 nm. Bovine serum albumin (BSA, 0.1 mg/mL) was used as the standard protein. All determinations were carried out in triplicate.

2.5. SDS-PAGE analysis

Sodium dodecyl sulfate-polyacrylamide gel (12.5%) electrophoresis (SDS-PAGE) was carried out according to the modified method of Laemmli [23]. Relative molecular mass was determined by performing SDS-PAGE analysis with molecular weight standards under reducing condition. The molecular weight standards used were phosphorylase b (97.4 kDa), bovine serumalbumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20.1 kDa) and lysozyme (14.3 kDa). The proteins were detected by staining the gel with 0.1% Coomassie brilliant blue R-250.

2.6. Reduction and alkylation of the protein

The protein (FtTI, 5 mg) was dissolved in buffer A, then reduced with 0.1 M dithiothreitol and alkylated (S-carboxymethylated) using 0.18 M iodoacetic acid as described by Crestfield [8]. It was then recovered by desalting on a column of Superdex G-75 (1.5 × 20 cm). The collected protein was lyophilized.

2.7. Determination of the disulfide bridge connectivity

To determine the disulfide bridge topology in FtTI, the method described by Stachowiak [40] was applied. The protein was dissolved in 250 mL of buffer A, to achieve the inhibitor concentration of 50 mM. Then 10 μg of thermolysin were added and the mixture