Food Chemistry 138 (2013) 1225–1232

Contents lists available at SciVerse ScienceDirect

Food Chemistry

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

Purification and characterisation of a novel chitinase from persimmon (*Diospyros kaki*) with antifungal activity

Jianzhi Zhang [a,1], Narasimha Kumar Kopparapu [b,1], Qiaojuan Yan [a], Shaoqing Yang [b], Zhengqiang Jiang [b,*]

[a] Bioresource Utilization Laboratory, College of Engineering, China Agricultural University, Beijing 100083, PR China
[b] Department of Biotechnology, College of Food Science and Nutritional Engineering, China Agricultural University, Beijing 100083, PR China

ARTICLE INFO

Article history:
Received 30 June 2012
Received in revised form 11 October 2012
Accepted 14 November 2012
Available online 24 November 2012

Keywords:
Persimmon
Chitinase
Purification
Antifungal activity
Chito-oligosaccharides

ABSTRACT

A novel chitinase from the persimmon fruit was isolated, purified and characterised in this report. The *Diospyros kaki* chitinase (DKC) was found to be a monomer with a molecular mass of 29 kDa. It exhibited optimal activity at pH 4.5 with broad pH stability from pH 4.0–9.0. It has an optimal temperature of 60 °C and thermostable up to 60 °C when incubated for 30 min. The internal peptide sequences of DKC showed similarity with other reported plant chitinases. It has the ability to hydrolyse colloidal chitin into chito-oligomers such as chitotriose, chitobiose and into its monomer *N*-acetylglucosamine. It can be used to degrade chitin waste into useful products such as chito-oligosacchaarides. DKC exhibited antifungal activity towards pathogenic fungus *Trichoderma viride*. Chitinases with antifungal property can be used as biocontrol agents replacing chemical fungicides.

1. Introduction

Chitin is insoluble linear polymer of β-1,4 linked *N*-acetyl D-glucosamine. It is present as an integral part of structural component of fungal cell walls, exoskeleton of arthropods and outer shell of crustaceans and nematodes (Dahiya, Tewari, Tiwari, & Hoondal, 2006). Chitinases (EC. 3.2.1.14) are the enzymes that can randomly hydrolyse the β-1,4 glycosidic bonds of chitin (Dahiya et al., 2006; Kaomek, Mizuno, Fujimura, Sriyotha, & Cairns, 2003; Santos, Cunha, Machado, & Gomes, 2004; Wang, Shao, Fu, & Rao, 2009). They are widely distributed in nature occurring in plants, animals, viruses, bacteria, fungi and insects. The roles of these enzymes among various organisms are diverse including defence, nutrient digestion, morphogenesis and pathogenesis (Dahiya et al., 2006; Wang et al., 2009). Chitinases possess wide range of applications in biotechnology, agriculture, medicine and environmental fields. They can be applied as biopesticides against pathogenic fungi and insects, and in production of chito-oligosacharides (Aam et al., 2010; Karasuda, Tanaka, Kajihara, Yamamoto, & Koga, 2003).

Depending on their mode of action, chitinases are classified as exo and endochitinases (Dahiya et al., 2006). Based on the amino acid sequence homology and their catalytic mechanisms the chitinases from various organisms are grouped into two glycoside hydrolases (GH) families 18 and 19 (Cantarel et al., 2009; Gomez, Allona, Casado, & Aragoncillo, 2002). Chitinases from bacteria, fungi, animals, plants and other organisms belong to GH family 18 (Dahiya et al., 2006; Kaomek et al., 2003), whereas GH family 19 chitinases exist mainly in plants and are reported to possess antifungal properties (Karasuda et al., 2003). Plants have evolved different modes of defence mechanisms to protect themselves from pathogens. Although chitin is not an integral part of plants, they do express chitinases that hydrolyse chitin (Wang, Ye, Chen, & Rao, 2012). In plants chitinases are involved in defensive mechanisms by acting against chitin containing fungal and insect pathogens (Taira et al., 2002; Karasuda et al., 2003; Santos et al., 2004; Wang et al., 2009). Chitinases are listed as the class of pathogenesis-related (PR) proteins which can be induced in number of ways either by viral, bacterial or fungal infection (Gomez et al., 2002; Shih, Khan, Jia, Wu, & Shih, 2001). *In vitro* experiments have shown that chitinases can inhibit the growth of many fungal species by causing lysis of the hyphal tip, which occurs presumably through the hydrolysis of chitin in the fungal cell wall (Taira et al., 2002; Wang et al., 2009). Due to their potential role in plant defence, chitinases possessing antifungal properties have received wide re-

Abbreviations: BSA, bovine serum albumin; CHES, N-cyclohexyl-2-aminoethane sulfonic acid; DKC, a chitinase from *Diospyros kaki*; DTT, dithiothreitol; EDTA, ethylenediaminetetracetic acid; GH, glycoside hydrolases; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TLC, thin layer chromatography.

* Corresponding author. Address: Post Box 294, China Agricultural University, No.17 Qinghua Donglu, Haidian District, Beijing 100083, China. Tel.: +86 10 62737689; fax: +86 10 82388508.
E-mail address: zhqjiang@cau.edu.cn (Z. Jiang).

[1] These authors contributed equally to this work.

0308-8146/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.foodchem.2012.11.067

search attention. Plant chitinases are constitutively present in certain tissues or reproductive organs such as seeds, roots, tubers, fruits and flowers. Till date, a number of chitinases have been reported from different seeds (Kopparapu, Liu, Fei, Yan, & Jiang, 2011a; Santos et al., 2004). However, there are few reports on chitinases from fruits such as grapes, papaya and pomegranate (Ano, Takayanagi, Uchibori, Okuda, & Yokotsuka, 2003; Chen et al., 2007; Kopparapu, Liu, Yan, Jiang, & Zhang, 2011b).

Persimmon is the edible fruit of the persimmon tree (*Diospyros kaki*), which belongs to *Ebenaceae* family. The fruit consists of a berry, as large as an apple, orange in colour, with soft, juicy pulp, sweet when it ripens (Anliker, Reindl, Vieths, & Wüthrich, 2001; Prandini & Marchesi, 1999). In China, it becomes ripe in late autumn and is usually eaten fresh by itself. Researchers have reported about the purification and characterisation of various enzymes such as β-galactosidase (Nakamura, Maeda, Mizuno, Koshi, & Nagamatsu, 2003) and leuco anthocyanidin reductase (Wang, Zhang, & Luo, 2010) from this fruit. However, there are no reports on chitinases from persimmon fruit (*D. kaki*). The present study describes the purification, characterisation and antifungal activity of a novel chitinase from persimmon.

2. Materials and methods

2.1. Materials

Persimmon fruits were obtained from local market. Q Sepharose FF, SP Sepharose FF, Sephacryl S-100 matrices were purchased from GE Life sciences, USA. Chitin was purchased from Sigma Chemical Co., USA. TLC silica gel sheets were purchased from E. Merck, Germany. Standard fungi samples were bought from Institute of Microbiology, Chinese Academy of Sciences (Beijing, China). All other chemicals and reagents used were of analytical grade unless otherwise stated.

2.2. Extraction and purification of a chitinase

Persimmon fruit was peeled and the pulp was taken in 50 mM sodium phosphate buffer (pH 7.4, 150 mM NaCl) and homogenised using a mixer grinder. The proteins were extracted for 4 h at 4 °C with constant stirring. After extraction, sample was filtered through the muslin cloth and the filtrate was centrifuged at 9500 rpm for 20 min. The resultant supernatant was considered as crude extract and was subjected to 80% ammonium sulfate precipitation. The precipitated proteins were recovered by centrifugation (9500 rpm for 20 min at 4 °C) and suspended in 20 mM Tris–HCl buffer (pH 8.5), dialysed against 20 mM Tris–HCl buffer (pH 8.5) for 16 h at 4 °C. The dialysed sample was centrifuged to remove the insoluble materials and loaded onto a Q Sepharose FF (1.0×8 cm) column, which was pre-equilibrated with 20 mM Tris–HCl (pH 8.5) buffer. The column was operated at 1 ml/min. The unbound sample was collected and the bound proteins were eluted by linear gradient of sodium chloride (0–150 mM NaCl) in 20 mM Tris–HCl buffer (pH 8.5).

The Q Sepharose FF unbound sample was dialysed against 20 mM sodium acetate buffer (pH 4.5) for 16 h at 4 °C. The dialysed sample was loaded onto a SP Sepharose FF (SP1) which was earlier equilibrated with 20 mM sodium acetate buffer (pH 4.5). Bound proteins were eluted by linear gradient of sodium chloride (0–100 mM NaCl) in 20 mM sodium acetate buffer (pH 4.5). SP Sepharose elution was concentrated and dialysed against 20 mM sodium acetate buffer (pH 4.0) and reapplied onto a SP Sepharose column (SP2) which was equilibrated with the same buffer. The unbound sample and the equilibration buffer wash were collected separately. Bound proteins were eluted by linear gradient of sodium chloride (0–100 mM NaCl) in 20 mM sodium acetate buffer (pH 4.0). Fractions showing chitinase activity were analysed on SDS–PAGE, homogenous fractions were pooled and concentrated by 10,000 Da cut off membrane, using Amicon stirred cell (Millipore, USA). The purified chitinase was used for further characterisation.

2.3. Preparation of the substrate (colloidal chitin) and enzyme assay

The substrate colloidal chitin 1% (w/v) was prepared according to the method described by earlier researchers, with minor modifications (Lee, Chung, Wi, Lee, & Bae, 2009). 10 g of chitin (Sigma Chemical Co., USA) was dissolved in cold concentrated HCl (200 ml) and placed at 4 °C for 24 h. The mixture was milled and added to 500 ml of 50% ethanol with rapid stirring. The precipitate was collected by centrifugation at 3000 rpm for 10 min at 4 °C and then washed with distilled water until the pH was neutral.

The chitinase activity was assayed using DNS method (Miller, 1959), by measuring the reducing end group *N*-acetylglucosamine (GlcNAc) using 1% colloidal chitin (w/v) as substrate. The standard reaction mixture consisting of 0.1 ml of suitably diluted enzyme and 0.1 ml of 1% (w/v) colloidal chitin (pH 4.5) was incubated at 50 °C for 30 min. After incubation, the reaction was terminated by addition of 0.3 ml dinitrosalicylic acid (DNS) reagent and heated in boiling water for 10 min. Then, the samples were rapidly cooled to room temperature by adding 0.25 ml of water and centrifuged at 8,000 rpm for 10 min. The absorbance of the supernatant was measured at 540 nm. One unit of chitinase activity was defined as the amount of enzyme that liberates 1 μmol of *N*-acetylglucosamine per minute under described conditions.

2.4. SDS–PAGE, protein quantification and isoelectric point determination

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed using 12.5% (w/v) acrylamide as resolving gel and 4.5% stacking gel as described by Laemmli (1970). Protein bands were visualised by staining with Coomassie brilliant blue R-250. The molecular weight standards were the low molecular weight SDS–PAGE marker (GE Life sciences, USA): rabbit phosphorylase b (97.0 kDa), bovine serum albumin (66.0 kDa), ovalbumin (45.0 kDa), bovine carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa).

The concentration of protein was quantified by the method of Lowry using bovine serum albumin as the standard (Lowry, Rosebrough, Farr, & Randall, 1951). For isoelectric point (p*I*) determination, the purified chitinase was submitted to Beijing Proteome Research Center (Beijing, China). The p*I* was estimated by electrofocusing, using Bio-Rad ReadyStrip™ IPG Strips (pH 3–10).

2.5. Native molecular mass determination and internal peptide sequences analysis

Native molecular mass of the purified chitinase was analysed by size exclusion chromatography using a calibrated Sephacryl S-100 column (1.0×100 cm). The column was equilibrated with 20 mM sodium citrate buffer (pH 4.5) containing 150 mM NaCl at a flow rate of 0.35 ml/min. The molecular mass standards used for calibration were phosphorylase B from rabbit muscle (97.2 kDa), bovine serum albumin (66 kDa), albumin from chicken egg white (44.28 kDa), α-chymotrypsinogen A: type II from bovine pancreas (25.6 kDa) and cytochrome *c* from equine heart (12.38 kDa). The retention times plotted as a function of the logarithm of molecular mass and the respective linear regression obtained were used for molecular mass determination.

To determine the partial amino acid sequence, the purified chitinase was subjected to SDS–PAGE. Protein band was excised from