International Journal of Biological Macromolecules 49 (2011) 894–899

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

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

1,3-β-Glucanase from *Vigna aconitifolia* and its possible use in enzyme bioreactor fabrication

Rakesh Mohan Kestwal [1], Dipali Bagal-Kestwal [1], Been Huang Chiang *

Institute of Food Science and Technology, National Taiwan University, No. 1, Roosevelt Road, Sec. 4, Taipei, Taiwan

ARTICLE INFO

Article history:
Received 31 May 2011
Received in revised form 31 July 2011
Accepted 3 August 2011
Available online 10 August 2011

Keywords:
Vigna aconitifolia
β-Glucanase
Purification
Characterization
Immobilization

ABSTRACT

Endo-1,3(4)-β-glucanase (EC 3.2.1.6) from *Vigna aconitifolia* sprouts was purified to 14.5 fold by gel filtration and ion-exchange chromatography. The enzyme was found to be a glycoprotein, its activity was Ca^{2+} dependent and specific for β-1,3 linkages in different polysaccharides. The K_m value of the enzyme was estimated to be 3.0 mg ml^{-1} for β-D-glucan as substrate. Circular dichroism studies revealed 8% α-helix, 48% β-pleated and 44% random coil in its secondary structure. Purified β-glucanase was then successfully co-immobilized with glucose oxidase in agarose–chitosan beads, showing better immobilization yield, operational range and stability as compared with the crude β-glucanase beads. The immobilized β-glucanase was successfully used for mini-bioreactor fabrication.

1. Introduction

Endo-1,3(4)-β-glucanase (systematic name: 3-(1→3;1→4)-β-D-glucan 3(4)-glucanohydrolase, EC 3.2.1.6) is an enzyme involved in endohydrolysis of (1→3) or (1→4) linkages in β-D-glucans [1]. In plants, the enzyme is involved in defense mechanism against fungal infections. If a plant is infected by fungus, the expression of β-(1→3)-D-glucanase is induced, to break down β-glucan present in fungal cell walls. The expression of β-(1→3)-D-glucanase in plants is upregulated by ethylene treatment or microbial infection and is down regulated if treated with phytohormones including auxin and cytokinin [2]. In addition, β-(1→3)-D-glucanase is involved in many other physiological functions including cell plate formation, pollen tube formation, seed germination, maturation, etc. [3]. The enzyme has applications in different biotechnological processes including protoplasts preparation, cell fusion, transformation, extraction of protein products, etc. [4]. It has also been used in the brewing industry as well as pharmaceutical industry for modification of β-glucans [5]. Pang et al. have purified and characterized an endo-1,3-β-glucanase from *Arthrobacter* sp. with crystallographic analysis [6]. But there are only few reports for β-glucanase purification from plants, especially sprouts.

Vigna aconitifolia (Jacq.) Maréchal (Moth Bean) is a hot-weather legume, belonging to family *Fabaceae*. It is one of the most drought tolerant pulse crop grown all over the world. The seeds are rich in protein (22–24%) and other nutritional components, which make them excellent supplement to cereal diets [7]. *V. aconitifolia* is also consumed after sprouting, which decreases trypsin inhibitor concentration and increases digestibility. In a preliminary study, an increased level of expression of β-glucanase was observed during sprouting of *V. aconitifolia*, hence the sprout was used as the source of enzyme.

Enzymes may be immobilized on various supports to enhance their stability and re-usability. Wide spread applications of enzymes in different industries is possible because of the immobilization [8]. Immobilized enzymes can be used in analytical devices, enzyme reactors, biosensors, etc. Purified and immobilized β-(1→3)-D-glucanase is important for the structural analysis of glucans [9]. The β-glucan, a macromolecule obtained from bacteria, fungi and plants, is recognized as a health-promoting compound which can protect the body against viral, bacterial and fungal infections, tumors, radiation effects, and stress-related immunosuppression [10,11]. Recently we reported a dip-strip based biosensor and electrochemical biosensor for β-glucan detection, using crude enzyme from *V. aconitifolia* sprouts [12,13]. Following the previous research, the objective of this study was to purify β-(1→3)-D-glucanase from *V. aconitifolia*, so that the chemical and physical characteristics of the enzyme could be determined.

Abbreviations: ADT, average daily temperature; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; FIA, flow injection analysis; IUBMB, International Union of Biochemistry and Molecular Biology; DEAE, diethyl amino ethyl; DNSA, dinitrosalicylic acid; 4-AAP, 4-amino antipyrine; PBS, phosphate buffered saline; BSA, bovine serum albumin; EDTA, ethylene diamine tetra acetic acid; GOD, glucose oxidase; CD, circular dichroism.

* Corresponding author. Tel.: +886 2 33664119; fax: +886 2 23697337.
E-mail address: bhchiang@ntu.edu.tw (B.H. Chiang).

[1] Both of them contributed equally to this work.

0141-8130/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijbiomac.2011.08.002

Further, we co-immobilize the purified enzyme with glucose oxidase in agarose–chitosan composite beads, and compared the characteristics of the free and immobilized β-(1→3)-D-glucanase. Attempt was also made to use the immobilized glucanase system for fabrication of mini-enzyme bioreactor and evaluate the feasibility of using this system for preparation of biosensor probes for β-(1→3)-D-glucan measurement.

2. Materials and methods

2.1. Materials and chemicals

V. aconitifolia (Moth bean) seeds were obtained from supermarket from Pune, India. Laminarin, Sephadex G-50, Sephadex G-100, DEAE-Sepharose CL-6B, β-D-glucan (from barley), zymosan, xylan, amylopectin, starch, carboxymethyl cellulose and standard protein markers for gel filtration were purchased from Sigma Aldrich (St. Louis, MO, USA). SeeBlue Plus2 pre-stained standard protein molecular weight marker for SDS-PAGE was obtained from Invitrogen (Carlsbad, CA, USA). All other chemicals were of reagent grade and used without further purification. Sunrise Basic ELISA reader (Tecan, Maennedorf, Switzerland) was used for taking readings of 96 well plates. Jasco J-810 spectropolarimeter was used for circular dichroism (CD) studies (Easton, MD, USA). Helios Alpha UV-Visible spectrophotometer (Thermo Spectronic, Cambridge, UK), Branson 5510 Ultrasonic instrument (Branson Ultrasonic Corp., CT, USA) and Gilson Minipuls peristaltic pumps (0.3 μl min^{-1} to 30 ml min^{-1}) (Gilson Medical Electronics, Villiers-le Bel, France) were also used in due course of studies.

2.2. Sprouting and extraction of glucanase

V. aconitifolia seeds were sprouted in a phytotron facility. Eight days old sprouts were washed gently with distilled water and the crude homogenate was obtained as previously described by Bagal-Kestwal et al. [12]. Dinitrosalicylic acid (DNSA) method was used for determination of β-glucanase activity at 540 nm and protein concentrations were determined as described by Kestwal et al. [12,14]. For estimation of enzyme activity, enzyme extract (400 μL) was mixed with 300 μL of phosphate buffer solution (0.2 M, pH 5.5) in a test tube. Then, 300 μL of β-glucan solution from barley (1 mg ml^{-1}) was added. After 10 min of reaction, the enzyme activity was terminated by addition of 500 μL DNSA reagent. The test tube was then kept in boiling water bath (100 °C) for 10 min. After cooling, the colored product was measured by spectrophotometer at 540 nm. One international unit is defined as the amount of enzyme which releases 1 μmol of reducing sugars equivalents (expressed as glucose) per minute using β-glucan as substrate at pH 5.5 and 40 °C.

2.3. Ammonium sulfate precipitation and gel filtration chromatography

The sprout aqueous extract was subjected to 45–65% ammonium sulfate precipitation for enzyme enrichment. The gel filtration was performed on Sephadex G-50 column (16 mm (Ø) × 700 mm (*L*)) in PBS. Each time 3.0 ml of extract was loaded and fractions of 8.0 ml were eluted every 15 min. Each fraction was checked for enzyme activity and protein concentration. Fractions with high specific activity were pooled together and concentrated by reverse dialysis.

2.4. DEAE-Sepharose CL-6B column chromatography

Concentrated β-glucanase was dialyzed against phosphate buffer (pH 7.5, 0.05 M) and applied onto a DEAE-Sepharose CL-6B column (16 mm (Ø) × 200 mm (*L*)). The column was washed with phosphate buffer (pH 7.5, 0.05 M) to remove the unbound protein and then eluted in stepwise manner with increasing concentration of NaCl (0.1–1.0 M) in phosphate buffer (pH 7.5, 0.05 M). The collected fractions (3 ml each) were analyzed for enzyme activity and protein concentration. Fractions with high specificity were pooled together, concentrated and once again loaded onto Sephadex G-50 column as final purification step. After the final step, the enzyme purity and homogeneity was checked using native PAGE, at pH 8.9 as well as pH 4.5.

2.5. Molecular weight determination

Molecular weight of the native (non-denatured) enzyme was determined using Sephadex G-100 column (16 mm (Ø) × 700 mm (*L*)). Alcohol dehydrogenase (150 kDa), bovine serum albumin (monomer) (66 kDa), carbonic anhydrase (29 kDa) and cytochrome C (12.4 kDa) were used as standard molecular weight markers. The molecular weight and subunit structure of the enzyme were also determined. For that SDS-PAGE (stacking gel 5% and separating gel 12%) was performed under reducing conditions, using pre-stained standard molecular weight marker (individual components: myosin (250 kDa), phosphorylase (148 kDa), BSA (98 kDa), glutamic dehydrogenase (64 kDa), alcohol dehydrogenase (50 kDa), carbonic anhydrase (36 kDa), myoglobin red (22 kDa), lysozyme (16 kDa), aprotinin (6 kDa) and insulin B chain (4 kDa)).

2.6. Influence of pH, temperature and substrate concentration on glucanase activity

The influence of pH on enzyme activity was studied at 37 °C. Different buffers used included, 0.2 M acetate buffer (pH 3.5–5.0), 0.2 M sodium phosphate buffer (pH 5.5–7.5) and 0.2 M Tris–HCl buffer (pH 7.5–9.5) in presence of β-glucan (from barley) (1.0 mg ml^{-1}). For studying the optimum temperature, the enzyme activity was assayed at temperatures from 30 to 100 °C in phosphate buffer (pH 5.5, 0.2 M). Thermostability test was also carried out, where the enzyme was pre-incubated for 30 min at different temperatures before addition of substrate. Michaelis constant K_m of the purified enzyme was also estimated using β-glucan as substrate. The effect of substrate concentration on the reaction rate was studied using β-glucan concentration from 1.00 mg ml^{-1} to 10.00 mg ml^{-1}.

2.7. Metal ion dependency and glycoprotein nature of enzyme

Purified enzyme was mixed with ethylene diamine tetra acetic acid (EDTA, 2.5 mM, 5.0 mM and 10 mM) (1:1, v/v) at pH 7.0 for 3 h, after which the enzyme activity was determined [15]. Reactivation of the EDTA inactivated enzyme was studied by mixing with different metal ions (Ca^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Hg^{2+} and Zn^{2+}) at 5 mM concentration for 15 min followed by determination of enzyme activity. Effect of individual divalent metal ions (5 mM) on enzyme activity in absence of EDTA was also checked in this study. The enzyme was also examined for glycosylation using phenol sulfuric acid method [14].

2.8. Substrate specificity studies

Substrate specificity for purified β-glucanase was done by analyzing the enzyme activity with different substrates. Enzyme activity with β-glucan (1.0 mg ml^{-1}) from barley was considered to be 100% of β-1,3-linkage for comparing with other substrates (1.0 mg ml^{-1} each). Different substrates used for this study included β-D-glucan (from barley), laminarin (from *Laminaria digitata*), zymosan (from *Saccharomyces cerevisiae*), xylan (from beech