Insect Biochemistry and Molecular Biology 39 (2009) 861–874

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

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

Purification, characterization and sequencing of the major β-1,3-glucanase from the midgut of *Tenebrio molitor* larvae

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ARTICLE INFO

Article history:
Received 18 June 2009
Received in revised form
8 October 2009
Accepted 12 October 2009

Keywords:
β-1,3-glucanase
Midgut
Laminarinase
Coleoptera
Fungi digestion

ABSTRACT

The major β-1,3-glucanase from *Tenebrio molitor* (TLam) was purified to homogeneity (yield, 6%; enrichment, 113 fold; specific activity, 4.4 U/mg). TLam has a molecular weight of 50 kDa and a pH optimum of 6. It is an endoglucanase that hydrolyzes β-1,3-glucans as laminarin and yeast β-1,3-1,6-glucan, but is inactive toward other polysaccharides (as unbranched β-1,3-glucans or mixed β-1,3-1,4-glucan from cereals) or disaccharides. The enzyme is not inhibited by high substrate concentrations and has low processivity (0.6). TLam has two ionizable groups involved in catalysis, and His, Tyr and Arg residues plus a divalent ion at the active site. A Cys residue important for TLam activity is exposed after laminarin binding. The cDNA coding for this enzyme was cloned and sequenced. It belongs to glycoside hydrolase family 16, and is related to other insect glucanases and glucan-binding proteins. Sequence analysis and homology modeling allowed the identification of some residues (E174, E179, H204, Y304, R127 and R181) at the active site of the enzyme, which may be important for TLam activity. TLam efficiently lyses fungal cells, suggesting a role in making available walls and cell contents to digestion and in protecting the midgut from pathogen infections.

1. Introduction

β-1,3-glucanases are enzymes that specifically hydrolyze β-1,3-glycosidic bonds in β-1,3-glucans like laminarin (β-1,3-glucan from the brown algae *Laminaria* sp), callose (from higher plants) or pachyman (from the fungus *Poria cocos*). They are also active against the β-1,3-1,6-glucan from yeast and fungi cell walls. They are endo- (glucan-endo-1,3-β-D-glucosidase, E.C.3.2.1.39) or exo- (glucan 1,3-β-glucosidase, E.C.3.2.1.58) enzymes. In a more recent classification of glycoside hydrolases based on hydrophobic amino acid clustering and sequence similarities, endo-β-1,3-glucanases from E.C.3.2.1.39 are present in families 16, 17, 55, 64 and 81, and exo-β-1,3-glucanases from E.C.3.2.1.58 are present in families 3, 5, 17 and 55 (Coutinho and Henrissat, 1999).

β-1,3-glucanases has been described as abundant in the digestive tract of insects of the orders Collembola, Trichoptera, Dictyoptera, Orthoptera, Isoptera, Coleoptera and Diptera (Terra and Ferreira, 1994). In spite of this, their properties are poorly known. In insects, only two laminarinases from *Periplaneta americana* (Dictyoptera) saliva (Genta et al., 2003), and one from *Abracris flavolineata* (Orthoptera) regurgitate (Genta et al., 2007) were purified to homogeneity, and characterized. Some enzymological details are known also for *Rhagium inquisitor* (Coleoptera) enzymes (Chipoulet and Chararas, 1984). Up to now, no data are available regarding to which glycoside hydrolase family they pertain. As β-glucanases are not present in vertebrates, they can be used as a target for insect control.

Tenebrio molitor (Coleoptera) is an important cosmopolitan pest of stored products (Richards and Davies, 1977). The properties and secretion of their β-glycosidases has already been studied (Ferreira et al., 2001, 2002, 2003).

Insect digestive laminarinases putatively play a role in hemicellulose (callose or cereal β) digestion in herbivores (Terra and Ferreira, 1994) or in the digestion of β-1,3-1,6-glucans from fungi in detritivores (Genta et al., 2003). In this paper, we describe the purification, characterization and sequencing of *T. molitor* digestive laminarinase (TLam). The data on specificity, action pattern, chemical modification, protein and cDNA sequencing indicate that TLam is

Abbreviations: TLam, *Tenebrio molitor* laminarinase; CFUs, colony-forming units; DPC, diethyl pyrocarbonate; EDC, N- (3-dimethylaminopropyl)-N′-ethylcarbodiimide; EDTA, ethylenediaminetetraacetic acid; GGE, 2-glyceroyl-3-glucosyl ethylglycol mixed acetal; NBS, N-bromosuccinimide; or-laminarin, periodate-oxidized and reduced laminarin; PG, 1 phenylglyoxal; pHMB, 4-(hydroxymercuri) benzoic acid; TEMED, N, N, N′, N′- tetramethylethylenediamine; TNM, tetranitromethane.

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doi:10.1016/j.ibmb.2009.10.003

a low processive β-1,3 endoglucanase and a member of glycoside hydrolase family 16 with a probable role in dietary fungal digestion.

2. Material and methods

2.1. Animals and chemicals

Stock cultures of *T. molitor* were maintained under natural photoregime conditions on wheat bran at 24–26 °C and 70–75% relative humidity. Fully-grown larvae of both sexes (each weighing about 0.12 g), having midguts full of food, were used.

Avicel was purchased from Merck (Darmstadt, Germany) and the other substrates were purchased from Sigma (USA). All chemical substances used were of analytical grade.

2.2. Preparation of samples

Larvae were immobilized by placing them on ice, after which they were dissected in cold 342 mM NaCl. For determination of enzymes in gut sections, tissues were homogenized in cold MilliQ water with the aid of a Potter–Elvehjem homogenizer with 10 strokes and centrifuged at 10,000 g for 10 min at 4 °C. The pellets were homogenized in cold MilliQ water using a micro tube homogenizer (Model Z 35, 997-1, Sigma, USA). The homogenates were stored at −20 °C until use without noticeable changes in the activities. Both pellets and supernatants were assayed.

For laminarinase purification, the midgut was pulled apart and homogenized as before in cold 20 mM imidazole buffer pH 7 containing 10 mM $CaCl_2$ and 10 mM phenylthiocarbamide (PTC). The homogenate was centrifuged as before, the supernatant was filtered through a PVDF membrane with a 0.44 μm pore (Millipore) and used immediately.

Wheat bran was homogenized in cold MilliQ water using a homogenizer model Skymsem TAR-02 (Siemsen, Brazil) at 10,000 rpm for 3 cycles of 30 s. The homogenate was sonicated with a Branson Sonifier 250, using three cycles of 30 s each (output 3) with 30 s intervals. The sample, after homogenization as described before, was centrifuged at 10,000 *g* for 10 min at 4 °C. The supernatant was passed through glass wool to be freed of fat.

2.3. Protein determination and hydrolase assays

Protein was determined with the silver method of Krystal et al. (1985), using ovalbumin as a standard. For this, samples were dialyzed for 3 h at room temperature against 20,000 volumes of 10 mM Tris, containing 10 mM Na_2CO_3, 0.75% Tween 20, with pH 10–12.

Hydrolase activity was determined by measuring the release of reducing groups (Noelting and Bernfeld, 1948) from 0.25% (w/v) laminarin (from *Laminaria digitata*), 0.125% (w/v) lichenan (from *Cetraria islandica*), 0.25% (w/v) carboxymethylcellulose (CMC), 0.25% (w/v) xylan, 0.125% (w/v) β-1,3-1,4-glucan from *Hordeum vulgare*, 1% (w/v) pachyman (from *P. cocos*) and 1% (w/v) Avicel (microcrystalline cellulose). The Avicel and pachyman suspensions were maintained under stirring during the whole assaying time. The release of glucose during the assays was determined with the Tris–glucose–oxidase method of Dahlqvist (1968). β-Glucosidase activity was determined following the release of p-nitrophenolate (Terra et al., 1979) from 5 mM p-nitrophenyl β-D-glucoside (NPβGlu), or the release of glucose from 5 mM laminaribiose, gentiobiose or cellobiose.

Unless otherwise specified, all substrates were assayed in 50 mM citrate-sodium phosphate pH 6.0 at 30 °C under conditions such that activity was proportional to protein concentration and to time. Controls without enzyme or without substrate were included. One unit of enzyme (U) is defined as the amount that hydrolyzes 1 μmol of bonds/min.

2.4. T. molitor laminarinase (TLam) purification

A sample of 2.5 mL of the midgut supernatant was applied onto a 5 mL EconoPac High Q ion-exchange chromatography column (EconoSystem, BioRad, USA) equilibrated with 20 mM imidazole buffer pH 7.0, containing 10 mM $CaCl_2$. After passing 20 mL of the last buffer through the column, elution was accomplished with 0–1 M NaCl gradient in the same buffer (100 mL) plus 20 mL of buffer with 1 M NaCl. The flow was 2 mL/min and fractions of 2 mL were collected. The more active fractions against laminarin were pooled (30–40, Fig. 1A), diluted ten times with the imidazole buffer above and the material was loaded onto a 1 mL Resource Q ion-exchange chromatography column (FPLC System, Pharmacia, Sweden) with a 150 mL Superloop (Pharmacia) and a flow of 4 mL/min. The column was equilibrated and washed (5 mL) with the same buffer. Elution was achieved with 0–1 M NaCl gradient in the same buffer (60 mL) plus 5 mL of buffer with 1 M NaCl. The flow was 1 mL/min and fractions of 0.4 mL were collected. Fractions more active against laminarin (37–43, Fig. 1B) were combined, $(NH_4)_2SO_4$ was added to attain a concentration of 1 M and the material was loaded onto a 1 mL Resource Phenyl hydrophobic-interaction chromatography column (FPLC) equilibrated with 50 mM MES buffer pH 6 containing 10 mM $CaCl_2$ and 1 M $(NH_4)_2SO_4$. After passing 5 mL of this buffer through the column, elution was carried out with 1–0 M $(NH_4)_2SO_4$ gradient (60 mL) and 5 mL of buffer without saline. The flow was 1 mL/min and fractions of 0.4 mL were collected. Fractions more active against laminarin (137–142, Fig. 1C) were pooled and applied onto a HR10/30 Superdex 75 gel filtration column (FPLC), equilibrated with 100 mM citrate-sodium phosphate buffer pH 6 containing 10 mM $CaCl_2$. Proteins were eluted with the same buffer (30 mL), with a flow of 1 mL/min, and fractions of 0.4 mL were collected. Fractions more active against laminarin (22–25, Fig. 1D) were combined and used as pure laminarinase (TLam).

In order to determine the molecular weight of TLAM, its elution from Superdex 75 was compared with the elution of the following standards: ribonuclease A (13.7 kDa), soybean trypsin inhibitor (21.5 kDa), ovalbumin (45 kDa), bovine serum albumin (66 kDa) and thyroglobulin (67 kDa).

2.5. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and detection of proteins and enzyme activity in the gel

SDS-PAGE was accomplished in gel slabs according to Laemmli (1970), as detailed in Ferreira et al. (2001). Staining for protein was done with the silver method of Blum et al. (1987). Molecular masses were calculated according to Shapiro et al. (1967). The following mass standards were used: lysozyme (14.4 kDa), soybean trypsin inhibitor (21.5 kDa), carbonic anhydrase (31 kDa), ovalbumin (45 kDa), bovine serum albumin (66 kDa) and phosphorylase b (97.4 kDa).

In gel assays were done after PAGE in 12% acrylamide gel containing 1% laminarin and the samples were not heated and no mercaptoethanol was added. The other conditions were as in SDS-PAGE. After electrophoresis, activity was detected according to a modification of the procedure of Pan et al. (1989) and Trudel et al. (1998), as described in Genta et al. (2003). For this, the gel slab was incubated in 20 mM 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer pH 6.0 containing 10 mM $CaCl_2$ for 15 min (buffer changes each 5 min). The gel was then incubated in a semi-dry system at 30 °C with the same MES buffer. After 15 min, the gel slab was