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Identification of a novel β-*N*-acetylhexosaminidase (Pcb-NAHA1) from marine Zoanthid *Palythoa caribaeorum* (Cnidaria, Anthozoa, Zoanthidea)

Djair S.L. Souza [a,b,c], Maria F. Grossi-de-Sa [a,d,*], Luciano P. Silva [a], Octavio L. Franco [d], José E. Gomes-Junior [a,b], Gustavo R. Oliveira [a,e], Thales L. Rocha [a], Cláudio P. Magalhães [a], Brener M. Marra [a,b], Maíra Grossi-de-Sa [a], Eduardo Romano [a], César Martins de Sá [b], Erich Kombrink [f], Arnubio V. Jiménez [a,b,g], Luiz R.D. Abreu [c]

[a] *Embrapa Recursos Genéticos e Biotecnologia, PqEB-Final W5 Norte-Cp02372, Brasilia, DF, Brazil*
[b] *Departamento de Biologia Celular, Universidade de Brasília, DF, Brazil*
[c] *Departamento de Bioquímica, UFRN, Rio Grande do Norte, Brazil*
[d] *Pós-Graduação em Ciências Genomicas e Biotecnologia, Centro de Análises Proteomicas e Bioquímicas, UCB, DF, Brazil*
[e] *Programa de Pós-graduação em Biologia Celular e Molecular, UFRGS, RS, Brazil*
[f] *Department of Plant–Microbe Interaction, Max Planck Institute for Plant Breeding Research, Carl-von-Linné-Weg 10, 50829 Cologne, Germany*
[g] *Universidad de Caldas, Facultad de Ciencias Agropecuarias, Manizales, Calle 65#26-10, Colombia*

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Abstract

β-*N*-Acetylhexosaminidases (EC 3.2.1.52) belong to an enzyme family that hydrolyzes terminal β-D-*N*-glucosamine and β-D-*N*-galactosamine residues from oligosaccharides. In this report, we purified a novel β-*N*-acetylhexosaminidase (Pcb-NAHA1) from the marine zoanthid *Palythoa caribaeorum* by applying ammonium sulfate fractionation, affinity chromatography on a chitin column, followed by two rounds of size exclusion chromatography. SDS–PAGE analysis indicated a single band protein of apparent homogeneity with a molecular mass of 25 kDa. The purified enzyme preferentially hydrolyzed *p*-nitrophenyl-2-acetoamide-2-deoxyamide-2-deoxy-β-D-*N*-acetylglucosamide (pNP-GlcNAc) and to a lesser extent *p*-nitrophenyl-2-acetoamide-2-deoxyamide-2-deoxy-β-D-*N*-acetylgalactosamide (pNP-GalNAc). Detailed kinetic analysis using pNP-GlcNAc resulted in a specific activity of 57.9 U/mg, a K_m value of 0.53 mM and a V_{max} value of 88.1 μmol/h/mg and k_{cat} value of 0.61 s^{-1}. Furthermore, purified Pcb-NAHA1 enzyme activity was decreased by $HgCl_2$ or maltose and stimulated in the presence of Na_2SeO_4, $BaCl_2$, $MgCl_2$, chondroitin 6-sulfate, and phenylmethylsulfonylfluoride. The optimum activity of Pcb-NAHA1 was observed at pH 5.0 and elevated temperatures (45–60 °C). Direct sequencing of proteolytic fragments generated from Pcb-NAHA1 revealed remarkable similarities to plant chitinases, which belong to family 18, although no chitinase activity was detected with Pcb-NAHA1. We conclude that β-*N*-acetylhexosaminidases, representing a type of exochitinolytic activity, and endo-chitinases share common functional domains and/or may have evolved from a common ancestor.
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Keywords: β-*N*-Acetylhexosaminidase; *Palythoa caribaeorum*; Chitin-active-enzymes; Inactivated chitinases

β-*N*-Acetylhexosaminidases[1] (EC 3.2.1. 52) are enzymes involved in the degradation of O-glycosides by catalyzing

* Corresponding author. Address: Embrapa Recursos Genéticos e Biotecnologia, PqEB-Final W5 Norte-Cp02372, Brasilia, DF, Brazil. Fax: +55 61 3340 3658.
E-mail address: fatimasa@cenargen.embrapa.br (M.F. Grossi-de-Sa).
URL: http://www.cenargen.embrapa.br/laboratorios/LIMPP/index.htm (M.F. Grossi-de-Sa).

[1] *Abbreviations used:* Pcb-NAHA1, *Palythoa caribaeorum* β-*N*-acetylhexosaminidase 1; pNP-GlcNAc, *p*-nitrophenyl-2-acetoamide-2-deoxyamide-2-deoxy-β-D-*N*-acetylglucosamide; pNP-GalNc, *p*-nitrophenyl-2-acetoamide-2-deoxyamide-2-deoxy-β-D-*N*-acetylgalactosamide.

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the hydrolysis of *N*-acetyl-D-hexosamine (glucosamine or galactosamine) residues from their non-reducing terminus [1–3]. These enzymes represent exoglycosidases that are classified into families 3 and 20 of the glycoside hydrolases (URL http://afmb.cnrs-mrs.fr/CAZY/). Their enzyme activity (biological function) is related to hydrolysis of oligosaccharides from glycoproteins [1,4], degradation of murein, a peptidoglycan found in bacterial cell walls [5], and glycosaminoglycans [6]. The β-*N*-acetylhexosaminidases can also act synergistically with chitinases in chitin depolymerization in protozoan, marine, and terrestrial fungi, as well as in diverse invertebrates, with the exception of sponges, some anthozoans and echinoderms [7].

Chitin is an insoluble polymer composed of repeat units of β (1, 4)-linked of *N*-acetyl-β-D-glucosamine, and represents the most abundant polysaccharide in the biosphere. It has been estimated that more than 10^{11} tons of chitin might be produced annually produced in marine waters, mostly from copepods [8]. However, chitin is almost absent from marine sediments, presumably as result of microbial degradation, which is responsible for maintaining equilibrium in carbon and nitrogen cycles [9–11]. Chitin contents differ across species, individuals, and even tissues [12], with their levels sustained by a balanced activity of enzymes, which synthesize and degrade chitin [13]. The generally accepted mechanism of chitin depolymerization and degradation involves enzyme families with different specificities and alternative sites of action along the chitin chain [14,15]. Initially, endochitinases cleave the substrate randomly, liberating different products, including soluble oligomers with variable molecular masses [16–18]. Extensive hydrolysis liberates smaller products, mainly *N*-acetylchitobiose (GlcNAc2) and *N*-acetylchitotriose (GlcNAc3), which serves as substrates for β-*N*-acetylhexosaminidases that act releasing *N*-acetylglucosamine monomers [19]. β-*N*-acetylhexosaminidase can be considered an exochitinase capable of cleaving the non-reducing terminal *N*-acetyl-D-hexosamine residues from *N*-acetyl-β-D-hexosaminides [20].

It is relevant to mention that chitinolytic enzymes are important for developmental processes in insects and crustaceans [12,21,22], as well for the nutrition of bacteria [23–26]. In fungi, chitinases are also involved in many developmental processes, including production and germination of spores, elongation and ramification of hyphae [27], and colonization of host tissues [28]. In plants, chitinases have been identified amongst the pathogenesis-related proteins that are induced by abiotic and biotic stresses, such as infection by pathogens, and thus have been associated with plant defense [60–62]. However they may also be related to developmental process, such as seed germination and fruit ripening [29–33]. So far, relatively few studies have described properties of chitinolytic enzymes from marine invertebrates [34–36]. Recently, the first cnidarian chitinase was cloned and characterize; and it was suggested that it was involved in host defense against fungal and nematode pathogens [37].

In this work, we described the identification of a novel β-*N*-acetylhexosaminidase from a marine zoanthid *Palythoa caribaeorum*. Occurrence of this particular β-*N*-acetylhexosaminidase in this chitinous organism suggests that it contains efficient enzymatic machinery for chitin synthesis and degradation. Although few reports have been published on *P. caribaeorum* [38–40], properties of chitin-degrading glycosidases or chitinases from this organism are unknown.

Materials and methods

Materials

Palythoa caribaeorum was collected on Santa Rita beach, Rio Grande do Norte, Brazil (5° 41′ 74″S, 35° 11′ 73″W). Material was maintained at –20 °C until extraction. pNP_NAG was powered from Sigma (St. Louis, MO, USA). Chondroitin 4-sulfate (C4-S) extracted from whale cartilage, chondroitin 6-sulfate (C6-S), extracted from shark cartilage and dermatan sulfate (DS) isolated from pigskin were acquired from Seikagaku Kogyo (Tokyo, Japan). Heparan sulfate (HS) was kindly provided by Prof. Dr. Carl Peter von Dietrich (UNIFESP/SP). LAOB Laboratories supplied purified heparin from bovine intestinal mucus and bovine lung (Barueri SP, Brazil) and UpJohn Co. (Kalamazoo, MI, USA), respectively. Bio-Gel A 1.5 m and 0.5 m columns were purchased from Bio-Rad Laboratories (Richmond, CA, USA). Chitin for affinity chromatography was extracted from red lobsters (*Panulirus argus*) shells according Carvalho and Garcia [64]. Fifteen grams of chitin were placed into a glass column and extensively washed (approximately 10 volumes) with 0.1 M sodium acetate buffer, pH 5.0. The chemicals $Na_2S_2O_3$, LiCl, $MgCl_2$, $CaCl_2$, $ZnSO_3$, $HgCl_2$, Na_2HPO_4, $Na_2H_2PO_4$ were purchased from VETEC (Rio de Janeiro/Brazil); and (Na_2SeO_4) from Sigma (Fair Lawn, NY, USA).

Extraction and purification of the β-N-acetylhexosaminidase

To achieve the objectives of this study, approximately 6 kg of *P. caribaeorum* were processed in a blender, using two volumes of 0.1 M sodium acetate buffer, pH 5.0. The homogenate was centrifuged at 27,000*g* at 4 °C for 30 min in order to separate the soluble fraction (crude extract/protein extract). In order to obtain the active fraction, ammonium sulfate was added at three different saturation levels (0–30%, 30–50%, and 50–80%). The pellet between 30% and 50% salt saturation was re-suspended in 2.0 ml of 0.1 M sodium acetate buffer, pH 5.0 and further submitted to dialysis (cut off 8 kDa) against the same buffer. The material was centrifuged as previously described and the rich fraction with highest β-*N*-acetylhexosaminidase activity (F50) was loaded onto the chitin column (2.8 × 17.1 cm), previously equilibrated with 0.1 M sodium acetate buffer, pH 5.0. Non-bound proteins were displaced with equilibration buffer. Bound proteins were

eluted with 0.1 M sodium acetate, pH 3.5. The β-*N*-acetylhexosaminidase fraction, eluted at pH 3.5, was precipitated with 90% saturated ammonium and centrifuged, under conditions described previously. The pellet containing the β-*N*-acetylhexosaminidase was dissolved, dialyzed against 0.1 M sodium acetate buffer at pH 5.0 and applied onto a Bio-Gel A 1.5 m (80 cm × 1.5 cm; void = 60 mL) column, equilibrated with 0.1 M sodium acetate buffer at pH 5.0. The fraction containing β-*N*-acetylhexosaminidase active was further applied onto a Bio-Gel A 0.5 m column (1.2 m × 1.5 cm; void = 90 mL) equilibrated with the same buffer. For analysis of enzyme purity, active-fractions from the samples from the Bio-Gel A 0.5 m were subjected to SDS–PAGE (12%) and analyzed according to Laemmli [41].

β-N-Acetylhexosaminidase assays

Samples from each purification step were incubated in a mixture containing 0.15 mM *p*-nitrophenyl-2-acetoamide-2-deoxy-β-D-*N*-acetylglucosamide in a final volume of 100 μL. The reaction was maintained at 37 °C for 60 min and stopped with 1 mL of 0.25 M NaOH. Activity was evaluated by spectrophotometry at 405 nm. For each assay, negative controls were included using 0.1 M sodium acetate buffer, pH 5.0. The reaction rate was calculated by utilizing the pNP molar absorption coefficient $1.73{\cdot}10^4\ M^{-1}\ cm^{-1}$ [42].

Chitinolytic assays

Chitinolytic activity was also evaluated by using 2.5 mg/mL chitin azure as substrate. Each assay was conducted with 60 μg of protein dissolved in 0.1 M sodium acetate buffer (pH 5.0) at 37 °C for 24 h. At the end of the assay, the mixtures were centrifuged and supernatant was measured at OD 575 nm. α-Amylase inhibitor with chitinolytic activity PvCAI, purified from common bean seeds was used as positive control [43]. In all assays, protein concentration was determined by the Bradford method [44], using bovine serum albumin (BSA) as standard.

Mass spectrometry and de novo sequencing

Protein bands containing the enzyme were excised from gels and digested into peptide fragments according to the method described by Shevchenko and Wim [45] with minor modifications. Gel fragments were pooled in a micro centrifuge tube and destained with a solution containing 50% acetonitrile and 24 mM NH_4HCO_3 (5 washes for 15 min). Fragments were dehydrated with 100% acetronitrile (15 min) and lyophilized. An in-gel digestion reaction was performed at 37 °C for 18 h with 50 mM NH_4HCO_3 solution containing Sequencing Grade Modified Trypsin (Promega V5111) at final concentration of 20 ng/μL. Samples of the digest (2 μL) were mixed with a saturated matrix solution of α-cyano-4-hydroxycinnamic acid (1:3) and spotted (0.5 μL) onto a MALDI target plate. Monoisotopic masses of the molecular components, ranging from *m*/z 600 to 6000 were determined by MALDI-TOF/MS using an UltraFlex II (Bruker Daltonics, Germany) controlled using the FlexControl 2.4.30.0 software. Molecular ions displaying appropriate signal were submitted to MS/MS analyses, which were carried out in the precursor ion fragmentation mode, using LIFT™ at a laser frequency of 50 Hz. The MS and MS/MS spectra were acquired in the reflector positive mode with external calibration, using the Peptide Calibration Standard—Starter Kit 4 (Bruker Daltonics). Resulting data were analyzed using FlexAnalysis 2.4 and MS/MS spectra were further interpreted manually (*de novo* sequencing) using PepSeq running under MassLynx 4.0 (Waters).

Immunoblotting

A protein sample (15 μg) from the active peak of the chitin column was diluted in 2× SDS buffer [125 mM Tris–HCl (pH 6.8), 4% SDS/10% glycerol/0.2% bromophenol blue/4% 2-mercaptoethanol] and subjected to SDS/PAGE on a 12.5% separating polyacrylamide gel under reducing conditions, according to Laemmli [41]. Proteins were then transferred to a nitrocellulose membrane by a semi-dry procedure, using a Pharmacia-LKB Multiphor II transfer system for a period of 45 min. Proteins in the membrane were detected using a rabbit anti purified basic (class I) chitinase from potato leaves [60,63]. A goat anti-rabbit immunoglobulin G coupled to horseradish peroxidase (Bio-Rad) was used as secondary antibody and the color developed using HRP color development reagent (Bio-Rad).

β-N-acetylhexosaminidase biochemical characterization

For determination of β-*N*-acetylhexosaminidase K_m and V_{max}, the purified enzyme was incubated at different concentrations of *p*-nitrophenyl-2-acetoamino-2-desoxi-β-D-*N*-acetylglucosamide (0–10 mM). The Michaelis–Menten constant (K_m) and maximum velocity (V_{max}) were calculated by double reciprocal transformation [46]. Optimum pH was examined after enzyme incubation (1 h) in different buffers: 0.1 M sodium acetate, pHs 4.0; 5.0, and 5.5), 0.1 M sodium phosphate, pH 6.8, and 0.1 M Tris–HCl, pHs 7.5 and 8.5. For optimum temperature determination, reaction mixtures were incubated at various temperatures (5, 25, 37, 45, 60, 70, and 80 °C) for 60 min in 0.1 M sodium acetate buffer, pH 5.0. For evaluation of effect of metal ion, β-*N*-acetylhexosaminidase was preincubated with 1 mM of $Na_2S_2O_3$, LiCl, $MgCl_2$, $CaCl_2$, $ZnSO_3$, $HgCl_2$. Residual activities were obtained by conducting enzyme assays under standard conditions. The effect of compounds such as *N*-ethylmaleimide, phenylmethylsulfonyl fluoride, iodoacetamide, diethylpyrocarbonate, and dycetone-2,4-pentadione on enzyme activity were also tested. The influence of glycans on enzymatic reactions namely (chondroitin 4-sulfate, chondroitin 6-sulfate, heparin, heparan sulfate,

galactan, dermatam sulfate, and fucoidan) and other sugars, such as fucose, fructose, glucose, galactose, mannose, glucuronic acid, maltose, lactose, and sucrose were also measured, with 4.5 μg of each saccharide.

Results

Purification of β-N-acetylhexosaminidase

A β-*N*-acetylhexosaminidase, designated Pcb-NAHA1, was purified from *P. caribaeorum* homogenates by three sequential chromatographic steps. As expected, a chitin affinity column was the most efficient purification step for Pcb-NAHA1, eliminating approximately 98% of unspecific proteins from the ammonium sulfate fraction F50 (Fig. 1; Table 1). Chromatographic elution profiles of Pcb-NAHA1 from two size-exclusion columns (Bio-Gel A 1.5 and 0.5 m) are shown in Fig. 1B and C. The whole procedures resulted in a more than 4800-fold purification of the enzyme, with a recovery of 25%, and a final specific activity of 57.9 U/mg proteins (Table 1). SDS–PAGE analysis revealed that Pcb-NAHA1 was purified and a single protein band with an apparent molecular mass of 25 kDa was detected (Fig. 2).

Substrate specificity

Several substrates were tested for conversion by Pcb-NAHA1, including *p*-nitrophenyl-2-acetoamide-2-deoxy-β-D-*N*-acetylglucosamide (pNP-GlcNAc), *p*-nitrophenyl-2-acetoamide-2-deoxy-β-D-*N*-acetylgalactosamide (pNP-GalNAc), and chitin. The purified enzyme was most active towards pNP-GlcNAc at 3 mM and when tested with pNP-GalNAc 3 mM showed about 40% activity. No activity was detectable when Pcb-NAHA1 was tested against chitin azure (Fig. 3).

Determination of K_m and V_{max}

An apparent K_m value of 0.53 mM, V_{max} of 88.1 μmol/min/mg, and k_{cat} of 0.61 s^{-1} were determined by testing Pcb-NAHA1 activity at variable concentrations of the substrate pNP-GlcNAc. The Lineweaver–Burk double reciprocal plot is presented in Fig. 4.

Effect of pH and temperature

Enzyme assays showed that Pcb-NAHA1 was active at a broad range of pH values, with maximum activity at pH 5.0 using pNP-GlcNAc as substrate (Fig. 5A). When tested at different temperatures, purified Pcb-NAHA1 showed a maximum activity at temperatures ranging from 45 to 60 °C, with inactivation at 70 °C or higher (Fig. 5B).

Effect of chemical compounds on enzyme activity

Several chemical compounds were evaluated for effect on Pcb-NAHA1 activity. The enzyme was completely inhibited by 1 mM $HgCl_2$ (Table 2). Other ions were also tested, inhibiting Pcb-NAHA1 activity only to a low extent, or even stimulating activity, such as $BaCl_2$, phenylmethylsulfonyl fluoride, and sodium selenate (Table 2). Different sugars and carbohydrates had no significant effect

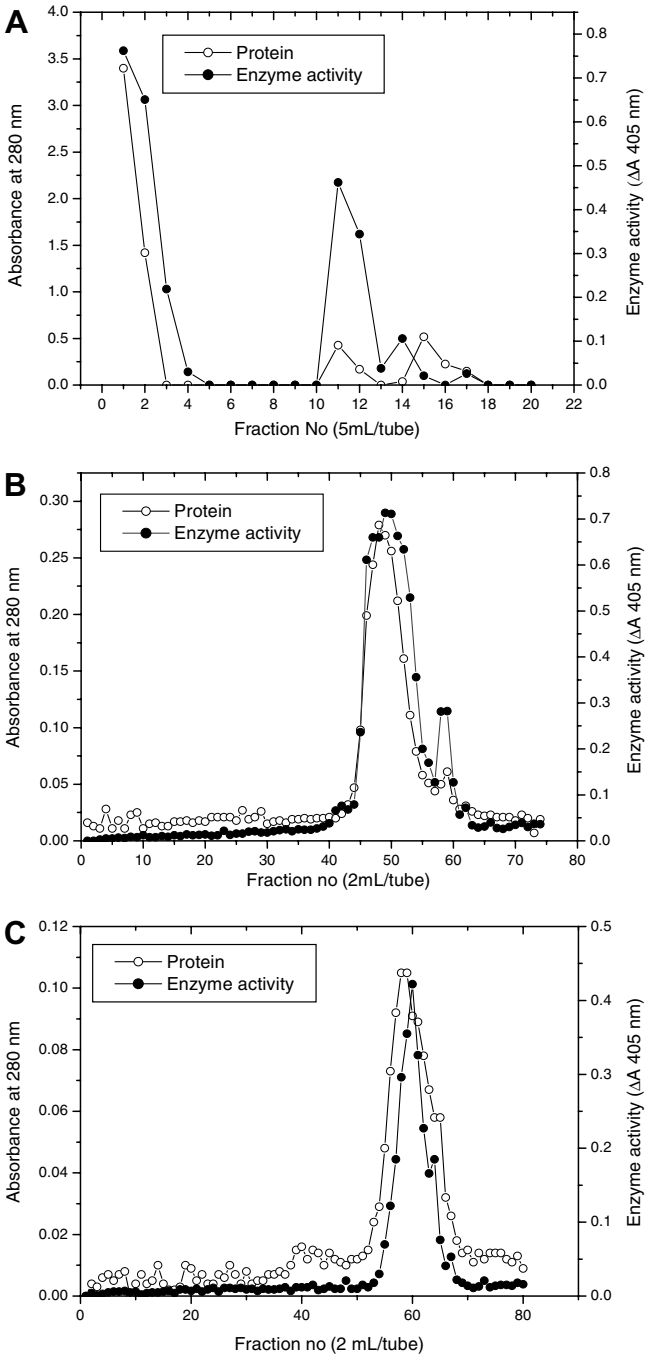

Fig. 1. Chromatographic profiles of Pcb-NAHA1. (A) The F50 fraction obtained after ammonium sulfate precipitation was applied to a chitin column (2.8 cm × 17 cm). (B) The chitin column pooled peak fraction eluted at pH 3.5 (11–13 fraction) was applied on Bio-Gel A 1.5 m. (C) The Bio-Gel A 1.5 m column peak fraction (44–54 fraction; void = 60 mL) was concentrated as described under Materials and Methods and applied onto a Bio-Gel A 0.5 m. The Bio-Gel A 0.5 m column peak fraction with enzyme activity (54–66 fraction; void = 90 mL) was concentrated and used for the Pcb-NAHA1 characterization.

Table 1
Summary of Pcb-NANA1 purification

Step	Total activity[a] (U)	Total protein[b] (mg)	Specific activity (U/mg)	Overall yield (%)	Purification (fold)
Crude extract	68.5	5900.0	0.0	100	1
F50	65.5	475.0	0.1	96	14
Chitin column	42.0	8.8	4.8	61	476
Bio-Gel A 1.5 m	57.6	2.5	23.5	84	2,350
Bio-Gel A 0.5 m	17.2	0.3	57.9	25	5,790

[a] One unit of enzyme activity was defined as the amount of enzyme which released 0.01 absorbance at 405 nm.
[b] Protein concentration was determined by the method of Bradford, using serum albumin as standard.

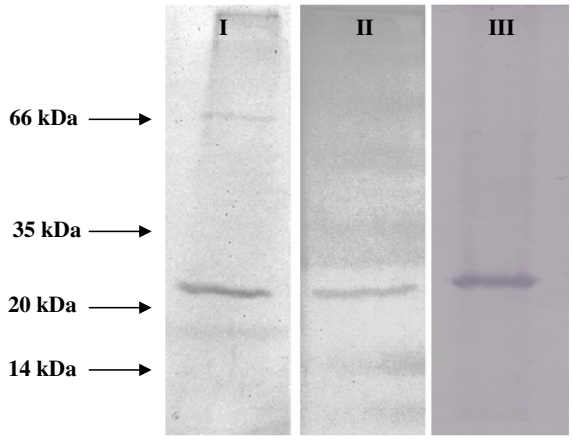

Fig. 2. SDS–PAGE of purified Pcb-NAHA1 from *P. caribaeorum*. Lane I, chitin fraction. Lane II, Bio-Gel A 0.5 m chromatography. Lane III, blot analyses of Pcb-NAHA1. Enzyme was detected in chitin column pooled peak fraction using a rabbit anti purified basic (class I) chitinase from potato leaves.

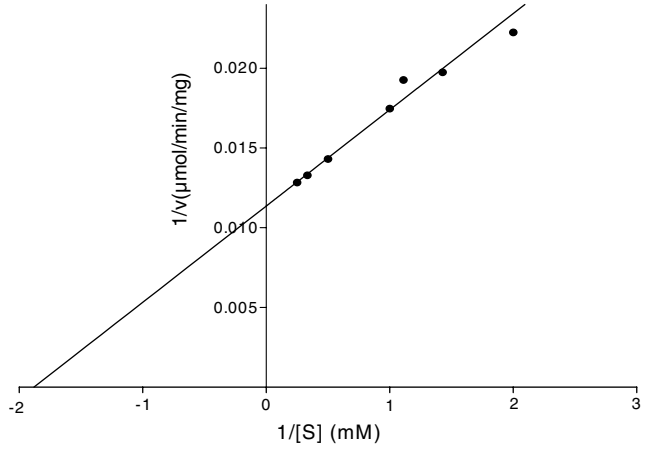

Fig. 4. Lineweaver–Burk graphic for the hydrolysis of pNP-β-D-GlcNAc by Pcb-NAHA1. The assay was performed at 37 °C and the conditions were 0.1 M sodium acetate buffer (pH 5.0) and different concentrations of pNP-β-D-GlcNAc.

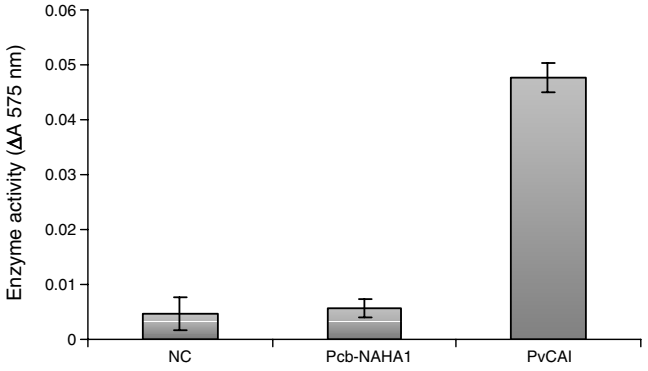

Fig. 3. Chitinase evaluation of Pcb-NAHA1 using chitin azure as substrate. Pcb-NAHA1 corresponds to β-*N*-acetylhexosaminidase purified with Bio-Gel A 0.5 m; NC corresponds to the negative control and PvCAI the positive control. The assays were carried out in triplicate.

on Pcb-NAHA1 activity, with the exception of maltose, which weakly inhibited Pcb-NAHA1 activity (33%) and chondroitin 6-sulfate, which stimulated enzyme activity by 35% (Table 3).

Sequence and Western blot analysis of Pcb-NAHA1

In order to establish the partial primary structure of Pcb-NAHA1, the enzyme was digested with trypsin and several fragments evaluated by sequencing *de novo* in a MALDI-TOF/TOF mass spectrometer. Among the identified peptides, the sequence GKSSSRPLGDATLDGLD FDIEVTQDYWDDLAR was 78.8% similar to proteins containing the hevein domain, such as the Hevamine-A precursor from *Hevea brasiliensis,* chitinases (EC 3.2.1.14), and bacterial lysozyme (EC 3.2.1.17) (Table 4). The apparent sequence similarity between Pcb-NAHA1 and chitinases was confirmed by protein blot analysis. Pcb-NAHA1 cross-reacted with a polyclonal antibody raised against basic (class I) chitinase from potato (Fig. 2).

Discussion

Purification of β-N-acetylhexosaminidase

Chromatography using insoluble (MPC)-chitin as affinity matrix has previously been described for the enrichment/purification of chitinases from fungi, such as *Verticillium chlamydosporium, V. suchlasporium,* and *Piromyces communis* [47,48]. Chitin columns have also been successfully used for the purification of chitinases from plant tissues such as bean and potato leaves, sorghum seeds, and from numerous other plants [33,48,59,61].

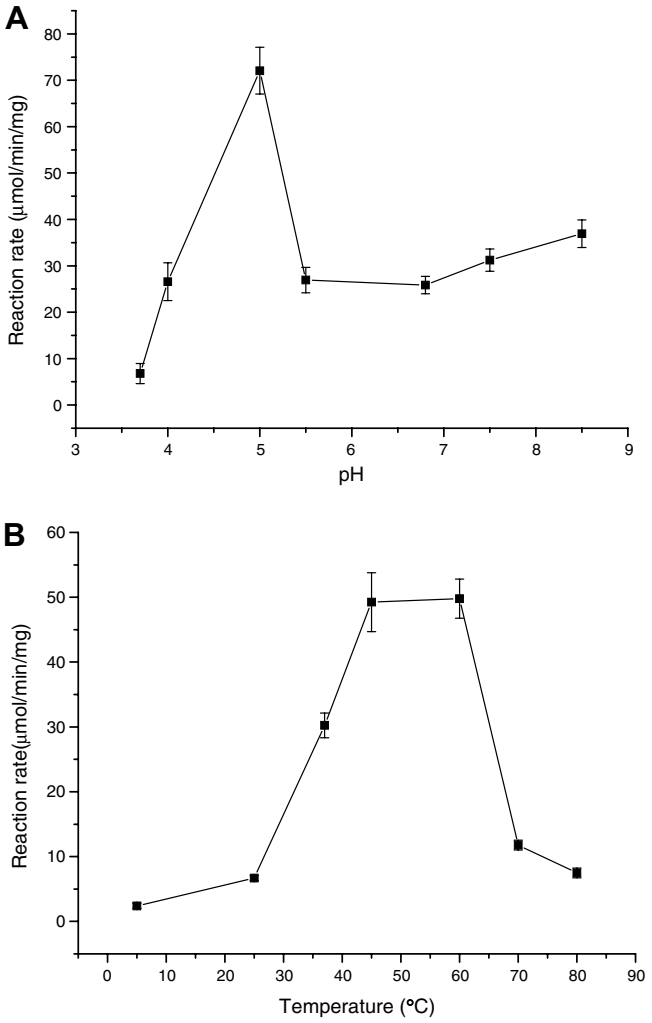

Fig. 5. The pH and temperature effects on Pcb-NAHA1 activity. pNP-GlcNAc was utilizes as substrate. Each assay was carried out in triplicate. Vertical bars indicate standard deviation.

Table 2
Ion and compound effects over Pcb-NAHA1 activity

Compounds	Relative activity (%)
$HgCl_2$	0
$CaCl_2$	86 ± 7
$CuSO_4$	90 ± 9
$BaCl_2$	140 ± 21
$MgCl_2$	117 ± 6
$ZnSO_4$	111 ± 8
$Na_2S_2O_3$	116 ± 3
LiCl	85 ± 12
Phenylmethylsulfonyl fluoride	146 ± 6
Diethylpyrocarbonate	108 ± 6
Iodoacetamide	84 ± 1
Dycetone-2,4-pentadione	116 ± 6
N-Ethylmaleimide	110 ± 10
Sodium selenate	145 ± 3

The enzyme was pre-incubated 30 min with each compound (1 mM) before the assay started. Results were presented as means ± standard deviation.

Table 3
Carbohydrate effects on Pcb-NAHA1 activity

Carbohydrate	Concentration	Relative activity (%)
Fucose	3 mM	101 ± 3
Fructose	3 mM	74 ± 1
Glucose	3 mM	91 ± 5
Galactose	3 mM	99 ± 0
Mannose	3 mM	81 ± 6
Maltose	3 mM	67 ± 8
Lactose	3 mM	80 ± 5
Sucrose	3 mM	95 ± 10
Glucuronic acid	3 mM	89 ± 2
Galactan	0.025 mg/mL	87 ± .5
Fucoidan	0.025 mg/mL	81 ± 7
Heparin	0.025 mg/mL	105 ± 1
Heparan sulfate	0.025 mg/mL	98 ± 0
Dermatan sulfate	0.025 mg/mL	101 ± 13
Chondroitin 6-sulfate	0.025 mg/mL	135 ± 5
Chondroitin 4-sulfate	0.025 mg/mL	119 ± 2

The enzyme was pre-incubated 30 min with each compound before the assay started. Results were presented as means ± standard deviation.

β-*N*-acetylhexosaminidases previously characterized from other sources have shown varying molecular masses, ranging from 50 to more than 100 kDa, including oligomeric forms [49]. However, a β-*N*-acetylhexosaminidase with an apparent molecular mass of 20 kDa was previously identified from the marine fungus *Phoma glomerata* [50].

Substrate specificity

Previous work has shown that in general β-*N*-acetylhexosamidases from family 20 are about four-times more active towards β-*N*-acetylglucosamides than β-*N*-acetylgalactosamides. By contrast, β-*N*-acetylhexosaminidases from family 3 are virtually inactive towards galactosyl substrates [51]. Apparently the enzyme is active towards chitin oligomers such as *N*-acetylchitobiose (GlcNAc2) and *N*-acetylchitotriose (GlcNAc3) released from chitin by the action of endochitinases [19]. Our results are in agreement with experiments described by Jin et al., who demonstrated that β-*N*-acetylhexosaminidases isolated from rice seeds were active toward pNP-GlcNAc and pNP-GalNAc, but unable to cleave chitin, glycol chitin and soluble chitosan [53].

Determination of K_m and V_{max}

For other β-*N*-acetylhexosaminidases, K_m values ranging from 0.006 to 1.65 mM have been reported [4,52,53]. Variations in the K_m can occur among different β-*N*-acetylhexosaminidases, which can be more or less specific towards the same substrate. Additionally, when different substrates are used with the same enzyme, a considerable variation in K_m values can be observed. For example, the *N*-acetyl-β-D-hexosaminidase from *T. vaginalis* showed a K_m value 1000 times lower when assayed against 4-MU-substrates than when using PNP-substrates [52].

Table 4
Alignment of tryptic fragment sequence of novel Pcb-NAHA1 with plant acidic chitinases

Primary access number	Sequence	Source	Identity(%)
Pcb-NAHA1	GKSSSRPLGDATLDGLDFDIEV-TQDYWDDLAR		
P23472	GKSSSRPLGDAVLDGIDFDIEHGSTLYWDDLAR	***H. brasiliensis***	78.8
Q9XF93	GHSTSRPLGDAVLDGVDFDIEGGNDQYWDDLAR	***F. ananassa***	75.8
Q84U85	GKSSPRPLGDAILDGIDLDIEGGTDLYWDDLAR	***R. glutinosa***	75.8
Q8RZ29	GSSSSRPLGDAVLDGIDFDIELGGAKFWDDLAR	***O. sativa***	72.7
Q71HN4	GKSLSRPLGDAVLDGIDFDIELGSTKYWDSLAR	***F. awkeotsang***	72.7
O22076	GKSSSRPLGDAVLDGIDFNIELGSPQHWDDLAR	***A. lyrata***	72.7
Q8LP09	GKSSSRPLGDAVLDGIDFNIELGSPQHWDDLAR	***A. lyrata***	72.7
O22074	GKSSSRPLGDAVLDGIDFNIELGSPQHWDDLAR	***A. gemmifera***	72.7
P19172	GKSSSRPLGDAVLDGIDFNIELGSPQHWDDLAR	***A. thaliana***	72.7
Q8LP04	GKSSSRPLGDAVLDGIDFNIELGSPQHWDDLAR	***A. gemmifera***	72.7
Q8LP05	GKSSSRPLGDAVLDGIDFNIELGSPQHWDDLAR	***C. wallichii***	72.7
Q8LP01	GKSSSRPLGDAVLDGIDFNIELGSPQHWDDLAR	***A. gemmifera***	72.7
Q8L4Q8	GKSSSRPLGDAVLDGIDFNIELGSPQHWDDLAR	***A. gemmifera***	72.7
Q1RYK5	GTSSSRPLGDAVLDGIDFDIEAGDGQYWDELAK	***M. truncatula***	69.7
P36910	GQSSTRPLGDAVLDGIDFDIESGDGRFWDDLAR	***B. vulgaris***	69.7
	* * ****** *** * ** ** **		

*Asterisks indicated residues totally conserved.

Effects of pH and temperature

The results related to optimum pH generated in this work are in agreement with those reported for β-*N*-acetylhexosaminidase extracted from rice seeds [53]. Optimum activity has been observed at pH values of 5.4, 4.7, and 5.7 for three β-*N*-acetylhexosaminidase isoforms from mungbean seedlings [54], and one β-*N*-acetylhexosaminidase purified from *Penaeus vannamei* was reported to be stable over a pH range of 4.2–10.0, with a maximum activity at pH 5.2 [7]. For other β-*N*-acetylhexosaminidases similar properties have been reported [53,13,55].

Effect of chemical compounds on enzyme activity

The effect of $HgCl_2$ on Pcb-NAHA1 activity is in accordance with properties of β-*N*-acetylhexosaminidase from rice [13,52,53]. Moreover, 0.1 mM $HgCl_2$ was able to inhibit the β-*N*-acetylhexosaminidase from green crab (*Scylla serrata*) [42]. Despite the increase of the Pcb-NAHA1 activity by phenylmethylsulfonyl fluoride (PMSF), no visible effect on enzyme activity was observed for the β-*N*-acetylhexosaminidase purified from rice seeds [53].

Sequence and Western blot analysis of Pcb-NAHA1

In spite of activity reported here, the, the enzyme exhibited a similar sequence to plant chitinases belonging to family GH18. These results suggest a possible evolutionary link between β-*N*-acetylhexosaminidases and chitinases. Despite the fact that the GH18 family constitutes a diverse range of proteins that are able to degrade chitin, proteins with high sequence similarity but no chitinolytic activity are common in this family [56]. Another example of such proteins is a new xylanase inhibitor (RIXI) from rice. This protein showed homology with GH18 chitinases, despite a lack of chitinase activity, and a clear function as xylanase inhibitor [57]. Proteins that exhibit chitinolytic activity without homology with chitinases have also been observed in other GH families, as described in APAP I and II isolated from the leaf beetle (*Gastrophysa atrocyanea*). These enzymes, classified as chitinases due to their activity, presented primary structures similar to glucanases of the GH48 family [58]. These data suggest that, in spite of the similarities found in sequences, especially as observed in high-throughput genome projects, it is therefore necessary to confirm activity assay results in order to obtain more precise information about the real biological role of individual glucosylhydrolases.

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