Constitutive Chitosanase from Bacillus Thuringiensis B-387 and its Potential for Preparation of Antimicrobial Chitooligomers

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Abstract

Ability of the insecticide strain *B. thuringiensis* var. *dendrolimus* B-387 to high constitutive production (3-12.5 U/mL) of extracellular chitosanase was found for first time. The enzyme was purified in 94-fold by ultrafiltration, affinity sorption and cation-exchanged chromatography and characterized biochemically. Molecular mass of the chitosanase determined using SDS-PAGE is 40 kDa. Temperature and pH-optima of the enzyme are 55°C and pH 6.5, respectively; the chitosanase was stable under 50–60°C and pH 4-10.5. Purified chitosanase most rapidly (V_{max}~43 μM/mL×min, K_M~0.22 mg/mL, k_{cat} ~ 4.79×10^4 s^{-1}) hydrolyzed soluble chitosan of deacetylation degree (DD) 85% by endo-mode, and no degraded colloidal chitin, CM-cellulose and some other glucans. Main reaction products of the chitosan enzymolysis included chitobiose, chitotriose and chitotetraose. In addition to small chitooligosaccharides (CHOs), studied chitosanase generated also low-molecular weight chitosan (LMWC) with average M_w in range 14–46 kDa and recovery 14–35%, depending of enzyme / substrate ratio and incubation temperature. Chitosan (DD 85 and 50%) oligomers prepared using crude chitosanase from *B. thuringiensis* B-387 indicated higher antifungal and antibacterial activities *in vitro* in comparison with initial polysaccharides. The findings evidence the chitosanase B-387 promising for bioactive CHOs preparation.

Introduction

Microbial chitinases (EC 3.2.1.14) and chitosanases (EC 3.2.1.132) are considered as one of excellent “tools” for enzymatic conversion of various chitin-containing wastes annually generated in enormous quantities under capture and processing of shellfish (Wang et al. 2011; Das et al. 2016; Pechsrichuang et al. 2018; Kaczmarek et al. 2019; Subramanian et al. 2020; Doan et al. 2020). According to the Food and Agriculture Organization of the United Nations (FAO) data, only aquaculture production of crustaceans and mollusks reached to more than 25 million tons in 2016 (The State of World Fisheries and Aquaculture 2018). Chitin as major structural polymer of exoskeleton, inner coverings and cell walls is found in most of invertebrate animals as well as in filamentous and yeast fungi, some unicellular algae and protozoan (Durkin et al. 2009; Abo Elsoud et al. 2019; Yadav et al. 2019; Garcia-Rubio et al. 2020; Jones et al. 2020). Therefore, chitin as unique structural analog of cellulose in animal and fungal life is also occurred ubiquitously, being as minimum second polysaccharide (after cellulose) on its world abundance with annual production more than 10^{10}-10^{11} tons only in aquatic ecosystems (Beier and Bertilsson 2013). Utilization of numerous chitin-containing sources, in one respect, is intended on overcoming of challenge caused by global pollution of marine waters and coastal areas with shellfish processing wastes (Yadav et al. 2019). On the other aspect, chitin is valuable material that highly promising in diverse fields of biotechnology, medicine, pharmacology, agriculture etc. (Khan et al. 2017). The main biotechnological role assigned to chitosanases in view of chitin wastes utilization purposes is limited destruction of chitosan, being deacetylated chitin derivative, for production of water-soluble and low-viscous bioactive oligomers (beginning DP 5–6) (Naqvi and Moerschbacher 2017). The products of extensively hydrolyzed chitosan including D-glucosamine (GlcN) and chitobiose ($GlcN_2$) forming under action of exo-chitosanases (EC 3.2.1.165) and some endo-chitosanases, respectively, are also valuable

compounds for medicine / pharmacology and promising starting material for chemical-enzymatic synthesis of higher chitooligosaccharides (CHOs) and their substituted derivatives (Jung and Park 2014). Enzymatic depolymerization of chitosan has many advantages over the physical-chemical methods of its destruction making possible controlled process under mild conditions of reaction mixture including temperature, pH and ionic strength parameters; in the absence of random modifications of functional groups and formation of undesired byproducts (Kaczmarek et al. 2019). As compared with other enzymes displaying chitosanolytic activity, chitosanases demonstrate highest specific action and effectively degrade the polysaccharide under minimal enzyme-substrate ratios resulting to sharp decrease of reaction products impurity with microbial proteins and metabolites (Pechsrichuang et al. 2018). This is crucial factor for preparation of CHOs with high-quality characteristics required especially for medicinal, pharmacological and food uses. At the same time, widespread biotechnological application of chitosanases is still restricted due to their production expense; therefore, the search of several approaches for its cost reduction remains one of current trends in explorations of these enzymes. In view of characterization of alternative microbial producers of chitosanase (including the enzymes with remarkable catalytic and physical-chemical properties), the capability of their cultivation in absence of such expensive inductor substrate as chitosan acquire significant importance. This work for the first time ever reports on constitutively produced chitosanase in member of *Bacillus thuringiensis*, entomopathogenic species that was practiced on a wide scale as biopesticide (insecticide) in crop production, horticulture and related fields (Ibrahim et al. 2010). Until the present, chitosanases from *B. thuringiensis* (Bt) are limitedly characterized in few studies if to compare with chitinases (Cruz Camarillo et al. 2004; Lee et al. 2007; Koboyashi et al. 2011). Moreover, their role in bacterial pathogenesis to various insect pests as well as involvement in antagonism against phytopathogenic fungi (in terms of antagonistic strains Bt) remains unexplored and far from full understanding. The potential of chitosanases from Bt for production of bioactive CHOs is also still studied enough fragmentarily and reported in few works, mainly, in view of chitosan conversion to oligomers with polymerization degree (DP) 3–7 (Olicón-Hernández et al. 2016; Kang et al. 2018; Santos-Moriano et al. 2018). This study is intended to purification and characterization of extracellular constitutive chitosanase from *B. thuringiensis* B-387, and evaluation of its potential for production of bioactive CHOs.

Materials And Methods

Reagents

The chitosan (Mw ~ 370 kDa, DD 85%) and partially N-acetylated chitosan (Mw ~ 200 kDa, DD ~ 50%) were obtained from the Laboratory of Biopolymer Engineering of Institute of Bioengineering of Federal Research Center “Fundamentals of Biotechnology” of Russian Academy of Sciences (Moscow, Russia). Flake crab shell chitin was provided by BioProgress Co. (Shchelkovo, Russia) and grinded in laboratory mill. Colloidal chitin was prepared from powdered crab shell chitin by modified method of Rodriguez-Kabana et al. (1983). Colloidal chitosan was prepared using the modified procedure (Helistö et al. 2001). N-acetyl-β-D-glucosamine, D-glucosamine, p-nitrophenyl-N-acetyl-β-D-glucosaminide (p-NP-GlcNAc), p-

nitrophenyl-N,N'-diacetyl-β-D-chitobioside (p-NP-$GlcNAc_2$), p-nitrophenol, sodium salt of carboxymethylcellulose, azokazein, laminarin and xylan from birch were purchased from Sigma Chemical Co., USA. CM-Sepharose Fast Flow was purchased from GE Healthcare (USA). The nutritional components of culture media (tryptone, yeast extract etc.) were obtained from Panreac (Barcelona, Spain) and HiMedia Laboratories Pvt. Ltd. (Mumbai, India). All other reagents, including components for SDS-PAGE, TLC, buffer salts etc. used in the study were qualified as analytical and high grade.

Microorganism cultivation and enzyme production

Chitosanase-producing strain, *B. thuringiensis* var. *dendrolimus* B-387 was obtained from All-Russian Collection of Microorganisms (VKM, Pushchino - Moscow, Russia) and available also in ATCC under number 19266 (Gordon et al. 1973). The strain is the base of the biopesticide "Dendrobacillin" effectively employed against Siberian silkworm larvae (Talalaev et al. 1971). The strain was maintained under 36°C on LB agar and on basal medium containing (g/L): $K_2HPO_4 \cdot 3H_2O$, 1; KH_2PO_4, 0.5; $(NH_4)_2HPO_4$, 0.5; $MgSO_4 \cdot 7H_2O$, 0.4; $CaCl_2$, 0.2; bactopeptone, 3; yeast extract, 3; corn steep liquor, 0.5; colloidal chitin (from crab shells), 5; agar, 16 (pH ~ 6.8 before autoclaving). For chitosanase production the strain was previously grown in LB broth overnight on Innova 40R shaker (New Brunswick Scientific, USA) under 36°C and 220 rpm. Grown bacterial cells were inoculated into LB broth (1:50, v/v) and cultivated in 250-mL Erlenmeyer flasks for 4 days at same conditions. Thereafter, the liquid culture was centrifuged (Sigma 2-16PK (Germany), 5°C, 6800×g for 30 min) for cell biomass removal, and clarified supernatant was subjected to further purification.

Purification of chitosanase and SDS-PAGE

On initial stage, the culture supernatant was concentrated by ultrafiltration on VivaFlow 200 (Sartorius, Germany) module with 10 kDa cut-off membrane. The enzyme concentrate obtained after this procedure was in further subjected to affinity sorption on colloidal chitosan (DD 85%, 5 mg/mL) as reported previously (Aktuganov et al. 2019). Finally, the chitosanase was purified by cation-exchange chromatography on CM-Sepharose Fast Flow using Biologic LP chromatographic system (Bio-Rad, USA). Chitosan-adsorbed fraction of the enzyme was loaded onto the column (2.5×10 cm) packed with CM-Sepharose previously equilibrated by four volumes of 50 mM tris-HCl buffer (pH 8.5). Unadsorbed proteins were removed from the sorbent with 75 mL of equilibrating buffer; in further the sorbent was successively washed by 0-500 mM of NaCl linear gradient (75 mL) and 500 mM of NaCl (100 mL) in the same buffer under flow rate 1.5 mL/min. Active fractions were pooled, desalted and concentrated using VivaPore 10/20 concentrators (Sartorius, Germany). Purity degree and molecular mass of the chitosanase were estimated by denaturating electrophoresis (SDS-PAGE) in 12.5% polyacrylamide gel with 0.1% of sodium dodecyl sulfate according to Laemmli (1970). PageRuler Broad Range unstained protein ladder (Thermo Scientific, USA) containing a mixture of eleven recombinant proteins ranging from 5 to 250 kDa was used as molecular mass standard. Separated proteins in gel were visualized using colloidal solution of Coomassie Brilliant Blue G-250 in the mixture of phosphoric acid (85%) – ethanol –

water (2:20:80 v/v) containing 8% (w/v) sulfate ammonium. In further gel was washed with deionized water for removal of background staining.

Measurement of chitosanase/chitinase activity and protein determination

Chitosanase and chitinase activities were determined by hydrolysis rate of soluble chitosan (Mw ~ 370 kDa, DD 85%) and colloidal chitin, respectively. For chitosanase assay, 0.5 mL of 0.5% (w/v) of chitosan solution in 100 mM sodium acetate (pH 6) was added to 1 mL of enzyme solution in the same buffer. Reaction mixture was incubated for 1 h under 50°C and stopped by 5 min heating in boiling water bath and centrifuged (Eppendorf Mini-Spin Plus, 9700×g for 5 min); reducing sugars concentration in 1 mL of supernatant was measured spectrophotometrically by Schales' method (Imoto and Yagishita 1971) using calibrating curve with D-glucosamine (GlcN) as standard (in range 100–200 μg/mL). The release rate of 1 μM equivalent of GlcN per min in 1 mL of reaction mixture at described conditions was expressed as 1 U of chitosanase activity. Chitinase was assayed by same protocol and proportions, except that 0.5% (w/v) of colloidal chitin suspension and 50 mM phosphate citrate (pH 6) were used as substrate and buffer solution, respectively. In that case, concentration of chitin degradation products was estimated on calibration curve (in range 150–250 μg/mL) with N-acetyl-D-glucosamine (GlcNAc). Reaction mixture containing equivalent volume of the enzyme solution incubated 1 h (50°C) without substrate was used as control.

Viscosymetric assay of soluble chitosans (DD 50 and 85%) hydrolysis by the chitosanase was evaluated using glass capillary viscometer of Ostwald type with capillary diameter 0.82 mm (Ecroskhim Co. Ltd., Russia) (Fig. S3).

Protein content in the enzyme samples was estimated by Warburg and Cristian method according to CHS protocol (2006) (Measuring protein concentration…).

Physical-chemical and kinetic characterization of chitosanase

Physical-chemical properties of the chitosanase including pH and temperature optima and stabilities, storage stability, effect of metal cations, salts, detergents and other additives were analyzed according to description in earlier published study (Aktuganov et al. 2019). Substrate specificity of the purified enzyme was assayed against 0.5% colloidal chitosan (DD 85%), 0.5% soluble chitosan (DD 50%), 0.5% colloidal chitin, 0.5% carboxymethyl cellulose sodium salt (CMC-Na), 0.2% laminarin, 0.5% xylan and 1% azokasein. Conditions for the enzyme reaction with mentioned substrates were analogous for standard chitosanase assay except for incubation time at use of laminarin (10 min), xylan (10 min) and azokasein (30 min). Protocols for determination of respective enzymatic activities were reported previously (Helistö et al. 2001). Relative activity of purified chitosanase toward listed substrates was expressed in percentage portion of its activity indicated against main substrate. The chitosanase was also tested for hydrolysis of chromogenic analogues of chitin dimer and trimer, p-nitrophenyl-N-acetyl-β-D-glucosaminide

(p-NP-GlcNAc) and p-nitrophenyl-N,N'-diacetyl-β-D-chitobioside (p-NP-GlcNAc)$_2$, respectively (Aktuganov et al. 2003).

Kinetic constants (K_M and V_{max}) calculated by measurement and using linearization equation $V_{max}=V_0 + [V_0/S_0]\times K_M$ according to Eisental and Cornish-Bowden method (1974). Kinetic of chitosan (DD 85%) destruction by purified chitosanase was analyzed for incubation period 10–240 min at standard conditions.

Thin-layer chromatography

Low-molecular weight products of chitosan enzymatic cleavage were assayed using thin-layer chromatography on Silica gel 60 F_{254} aluminum sheets (10×20 cm) (Merck, Germany). Purified chitosanase (44 μg ~ 0.42 U) was diluted 10-fold by deionized water and incubated with equivalent volume of 0.5% (w/v) soluble chitosan (DD 85%) for 10, 30, 60 and 240 min under 50°C and pH 6. Samples of reaction mixture taken at different time intervals were applied (1–2 μL) on TLC sheets. The solution including n-BuOH : NH_4OH(28%) : MeOH : H_2O (5:4:2:1) was used as mobile phase in TLC chamber. After migration of solvent front to upper edge of TLC sheet it was dried and treated with 0.25% (w/v) of ninhydrin solution in acetone for staining of mobile oligomers. A commercial mixture including chitosan oligomers with polymerization degree n = 2–6 ("MedChemExpress" LLC, USA) as well D-glucosamine ("Sigma", USA) was used as standard.

HP-SEC of LMWC released under chitosan enzymatic hydrolysis

Crude chitosanase complex was added to 2% (w/v) chitosan (DD 85%) solution in 50 mM sodium acetate buffer (pH 6) at volume ratios 1:600, 1:120 and 1:60 (enzyme – substrate ratios were 0.001, 0.005 and 0.012 U/mg, respectively) and incubated for 60 min under 50°C (for ratios 1:600 and 1:60) or 70°C (for 1:120). Residual substrate (LMWC) was precipitated by adding of 2M solution of NaOH (1:1, v/v), centrifuged, washed by deionized water to neutral reaction and re-dissolved in 50 mM sodium acetate buffer (pH 6). Prepared solution was lyophilized; dry residue was weighted and again re-dissolved in deionized waster for further analysis. Average molecular mass (M_w), number-average molecular mass (M_n) and polydispersity index (M_w/M_n) of LMWC were assessed by high performance size-exclusion chromatography (HP-SEC) using liquid chromatograph S-2100 (Sykam, Germany) with the PolySep-GFC-P 4000 column (7.8×300 mm) (Phenomenex, USA) and pre-column (5.0×2 mm). The chromatography was performed under flow rate 0.5 ml/min, temperature 30°C and pressure 2 MPa. The mixture of 50 mM acetic acid and 150 mM ammonium acetate (pH 5.1) was used as eluent. The column was calibrated using dextran standards (Mw 1.08; 4.44; 9.89; 43.5; 66.7; 123.6 and 196.3 kDa) (Sigma, USA). Chromatogram analysis was carried out using software Chrom&Spec v. 1.6 (Ampersand Inc., Russia).

Antibacterial and antifungal assays

Bactericidal and fungicidal activities were assayed for mixture of all CHOs molecules accumulated in reaction mixture after 60 min treatment of chitosans with DD 85% and 50% by crude enzyme complex of *B. thuringiensis* B-387. Reaction mixture was centrifuged, sterilized by filtration trough bacterial syringe filters GVS (USA) with pore size 0.20 μm and lyophilized in sterile vial. Weighted quantities (25-1000 μg) of prepared dry preparations of CHOs were dissolved in MQ-water immediately prior to experiment performance. Antibacterial/antifungal assays were carried out in 96-well polystyrene cultural plates (Corning Inc., USA) with test medium volume 200 μL per well. CHOs activity was determined as growth inhibition (%) of tested bacterial and fungal cultures, respectively, in LB and potato-dextrose broths. Optical density of grown tested microorganisms was measured using plate reader EnSpier (Perkin-Elmer, USA) at 600 nm after 24-h and 72-h of pre-cultivation (28°C) of bacteria and micromycetes, respectively. Phytopathogenic fungal species as well as non-beneficial bacterial strains used as tested microorganisms were obtained from All-Russian Collection of Microorganisms (VKM) and own microbial collection. Bacterial cultures were pre-cultivated for overnight in LB broth under 30°C and 220 rpm. Grown bacteria were then diluted by sterile LB broth to optical density value A_{600} = 0.05 and 100 μL of prepared bacterial suspensions were filled into wells. Suitable quantities of tested CHOs were added to the aliquots and adjusted to final volume (200 μL) with sterile LB broth. Fresh 6 days-old fungal cultures were taken for preparation of spore suspension (10^5 spores per mL) in sterile potato-dextrose broth using counting chamber. Other manipulations for antifungal assay were analogous to these in mentioned above antibacterial test. Growth inhibition (GI) degree (%) of test-microorganisms was calculated according to formula: $GI = [(A_{600K} - A_{600E})/ A_{600K}] \cdot 100$, in which A_{600K} and A_{600E} are values of optical density for microbial cultures in control and experimental samples, respectively. CHOs doses were substituted with same volumes of sterile physiological solution in control variants. Positive control samples included same concentrations of original high-molecular weight chitosans (DD 85% and 50%). Minimal aliquots of CHOs causing no less than 50% bacterial / fungal growth reduction were taken as minimal inhibiting concentrations (MIC).

Statistical analysis

The experiments for evaluation of fungicidal/bactericidal activities of CHOs as well as viscosimetric assay and enzymatic activities' measurements were performed in triplicate; the data are presented as mean values with standard deviation (SD). The differences between compared samplings were considered significant at $p < 0,05$.

Results

Culture growth and chitosanase production

Chitosanase production in LB broth reached the maximal level (> 3 U/mL) to 48 h of cultivation insignificantly increasing later. Bacterial growth demonstrated maximal value at 24 h (~ 6.6×10^8 CFU/mL) sharply declining to 4 day of fermentation (Fig. 1). This behavior probably can be resulted from autolysis of bacterial cells in course of sporulation that was registered earlier for representatives of the

species (Chen et al. 2018). During fermentation pH value of culture liquid increased from 6.5 to 9, however, it had no visible effect on chitosanase activity. Along with chitosanase production, strain B-387 demonstrated also relatively slight basal chitinase activity (~ 0.1 U/mL) under cultivation in LB broth (Fig. 1). Its chitinase production increased in 3-5-fold in the medium supplemented with 0.5% (w/v) of colloidal chitin (data are not presented). Hence, it seems that chitinases of B-387 are inducible enzymes as contrasted with chitosanases.

Purification of chitosanase

The chitosanase from culture supernatant of *B. thuringiensis* B-387 was reproducibly purified using three sequential procedures to electrophoretically homogenous state. Main steps of the purification are summarized in Table 1. Ultrafiltration procedure resulted commonly to 10-fold increment of enzyme activity in concentrate volume (U/mL), while specific chitosanase activity increased approximately by three times. Affinity sorption demonstrated reversible binding of the enzyme by colloidal chitosan (5 mg/mL, DD ~ 85%) ranged from 70 to 75% of its total initial activity in crude preparation (data are not presented). Maximal effectiveness of the chitosanase purification was reached by means of cation-exchange chromatography on CM-Sepharose. The sharp activity peak of the enzyme was revealed within minor protein peaks eluted by linear gradient of 0-500 mM M NaCl, while non-adsorbed proteins of major peaks no indicated chitosanase activity (Fig. 2a). Final purification degree of the chitosanase was about 94-fold; molecular mass of the purified enzyme was approximately 40 kDa according to SDS-PAGE (Fig. 2b, line 3). The chitosanase fraction non-adsorbed on colloidal chitosan after affinity sorption step was also effectively purified on CM-Sepharose under same conditions to electrophoretically homogenic state and revealed as protein band with same molecular mass (Fig. 2b, lines 4–5).

Table 1
Purification of the chitosanase from *B. thuringiensis* B-387

Purification stage	Total protein, mg	Total chitosanase activity, U	Specific chitosanase activity, U/mg	Enzyme recovery, %	Purification degree (-fold)
Culture supernatant	4722.30	1619.68	0.34	100	1
Ultrafiltration on VivaFlow 200 module, ≤ 10 kDa	822.7	931.32	1.13	57.5	3.3
Affinity adsorption on colloidal chitosan (0.5%)	204.79	730.48	3.57	45.1	10.4
Cation-exchange chromatography on CM-Sepharose Fast Flow	4.57	147.39	32.25	9.1	94

Physic-chemical properties of purified chitosanase

Influence of pH on the chitosanase activity and stability was assayed toward soluble chitosan (DD ~ 85%) under limitedly wide range of values (pH 3-10.5). As expected, the purified enzyme most actively functioned in range of pH 5-7.5 with optimum at pH 6.5 (Fig. 3a). Furthermore, unlike with many bacterial chitosanases, the enzyme retained noticeable activity at pH 8–9, when chitosan partially settles out forming suspension systems. The enzyme demonstrated stability under pH 5-10.5 completely maintaining original activity after 24 h pre-incubation (Fig. 3a). Temperature dependence of the enzyme activity is characterized with pronounced optimum at 55°C, however, the chitosanase was highly active also at 60–65°C and retained more than 20% of its activity at 75°C (Fig. 3b). The enzyme was moderately stable under 50–60°C sustaining approximately 75 and 50% of original activity, respectively, after 1 h incubation in absence of substrate. The crude chitosanase demonstrated satisfactory stability under long-term storage. Common storage of the culture supernatant during 7 months in non-sterile vessel at 5°C was little different on its residual activity (93–100%) from other approaches including use of sterilized containers, addition of preservative agent and lyophilization procedure (Fig. S1).

The purified enzyme was assayed in presence of limited number of salts and chemicals (Table 2). None tested bivalent metal salts displayed activating action to the chitosanase including such potential co-factors and activators as Mg^{2+}, Ca^{2+}, Fe^{2+}, Mn^{2+} and Zn^{2+}. Most strong inhibitors among metal cations were Hg^{+} (at 10 mM and 1 mM), Cd^{2+} (10 and 1 mM), Zn^{2+} (10 mM) and Ag^{+} (10 and 1 mM). Among other verified chemicals only sodium lauryl sulfate (10 mM) expectedly exerted severe inhibition, whereas non-ionic surfactants, urea and polyglygols did not have visible effect except for tween-80 (10 mM) that solely displayed distinct activating action (Table 2). Sodium chloride distinctly (by 40–50%) suppressed the enzyme's activity only under concentrations higher than 1 M (Fig. S2). This salt is capable to improve chitosan solubilization at some concentrations range thereby favoring to increase by times the activity of some hydrolases as for instance the exo-chitosanase from *Penicillium* sp. IB-37-2 (Aktuganov et al. 2019). However, such effect was not observed for the chitosanase of Bt B-387.

Table 2
Effects of metal cations and some chemicals on activity of the chitosanase from *B. thuringiensis* B-387

Metal cations (concentration)	**Relative effect, %**
Zn^{2+}, Hg^{+}, Cd^{2+} (10 mM each)	Complete suppression (100%)
Ag^{+} (10 mM), Cd^{2+} (1 mM), Fe^{2+}(10 mM and 1 mM)	Strong suppression (84–90%)
Ag^{+} and Hg^{+} (1 mM each)	Significant suppression (60–64%)
Ca^{2+}(10 mM and 1 mM), Zn^{2+} (1 mM)	Slight or moderate suppression (8–20%)
Mg^{2+}, Mn^{2+}(10 mM and 1 mM each)	No visible effect
Detergents and other chemicals (concentration)	
SDS (10 mM)	Strong suppression (84–90%)
SDS (1 mM)	Moderate suppression (30%)
Tryton X-100 (10 mM)	Slight suppression (8%)
Urea, PEG-300 (10 mM and 1 mM each), Tryton X-100 (1 mM)	No visible effect
Tween-80 (1 mM)	Slight activation (5–10%)
Tween-80 (10 mM)	Noticeable activation (to 40%)

Kinetic characteristics and substrate specificity

The purified chitosanase most actively hydrolyzed soluble chitosan with DD 85%. Kinetic constants (K_M and V_{max}) calculated by measurement and using linearization equation $V_{max}=V_0 + [V_0/S_0]\times K_M$ according to Eisental and Cornish-Bowden method (1974) amounted 0.22 mg/mL and approximately 43 μM/mL×min, respectively (Fig. 3, c-d). Empirical calculation from substrate concentration dependence curve resulted to similar value of K_M but to lesser V_{max}~34.987 μM/mL×min. Nearby value of specific V_{max} was obtained from the enzyme concentration dependence curve (Fig. 3c). According to this, catalytic constant k_{cat} was 4.792×10^4 s^{-1}, k_{cat} / K_M =1.948×10^5 $mg^{-1}\times s^{-1}$.

Among other substrates, colloidal chitosan (DD 80–85%) and partially N-acetylated soluble chitosan (DD 50%) were more slowly hydrolyzed by the chitosanase (V_{max} was 19.08 and 12.56 μM/mL×min; K_M was 0.98 and 3.19 mg/mL, respectively) (Table 3). Viscosity kinetic assay also indicated lower depolymerization rate of chitosan with DD 50% by the enzyme (Fig. S3). The purified enzyme no degraded colloidal chitin, chromogenic analogues of chitooligosaccharides p-NP-GlcNAc and p-NP-$(GlcNAc)_2$, Na-CMC, laminarin, xylan and azokazein (Table 3).

Table 3
Substrate specificity of the chitosanase from *B. thuringiensis* B-387

Substrate	Relative activity, %
Soluble chitosan (DD 85%)	100
Colloidal chitosan (DD 85%)	44.4
Soluble chitosan (DD 50%)	29.2
Colloidal chitin	0
Laminarin	0
CM-cellulose (sodium salt)	0
Xylan	0
Azokazein	0
p-NP-GlcNAc	0
p-NP-(GlcNAc)	0

Main enzymatic reaction products of extensively hydrolyzed chitosan DD 85% (60–240 min) include chitobiose, chitotriose, chitotetraose and chitopentaose according to TLC assay (Fig. 4). Short incubation (10 min) resulted to rapid decrease of the substrate viscosity with formation of mixture containing longer chitooligomers (n = 4–7 etc.). Thus, the chitosanase of Bt B387 demonstrates typical pattern for endo-mode of chitosan splitting.

Chitosan conversion by the chitosanase into water-soluble chitooligomers

Crude enzyme complex of Bt B-387 converted more than 95% of chitosan (DD 85%) into water-soluble oligomers within 60 min incubation at enzyme-substrate ratios 0.01–0.04 U/mg. Preliminary HP-SEC analysis of LMWC prepared by 60-min enzymatic hydrolysis of 2% chitosan (at enzyme / substrate ratio 0.001 U/mg) indicated its average molecular weight about 46 kDa with polydispersity index (PDI) 2.03 (Fig. 5a). Ten-fold decrease of enzyme-substrate ratio (to 0.012 U/mg) resulted to predominant generating of LMWC with average molecular weight 16.7 kDa and PDI 1.55 (Fig. 5c). Interestingly, that increase of incubation temperature from 50 to 70°C leaded to formation of LMWC with Mw_a~14.3 kDa (PDI 1.62) under midline enzyme-substrate ratio (0.005 U/mg) (Fig. 5b). Recovery of LMCW by dry weight under described conditions reached 29.2, 35 and 14.6%, respectively. The findings demonstrate the probability of responsive approach in potential application of the Bt B3-87 chitosanase for diverse challenges including preparation of LMCW with variable molecular weight or small chitooligosaccharides with DP = 2–5.

Fungicidal and antibacterial activity of chitooligosaccharides prepared using the chitosanase of Bt B-387

This work analyzed preliminary fungicidal activity of total CHOs mixtures formed after 60-min hydrolysis of 1% chitosans with DD 85 and 50% by the crude chitosanase from Bt B-387 (~ 0.048 U/mg). It is worth to note, that antifungal effect of assayed hydrolytic products (including LMWC) deviated differently from original polymer influence depending of tested fungal species. On the one hand, inhibition degree of *F. oxysporum* and *R. solani* was definitely decreased in both chitosans enzymatic hydrolysates under concentration range 25–150 μg/mL (Table 4). It is no surprise, taking into account relatively extensive hydrolysis of chitosan in this experiment. Nevertheless, increase of fungicidal effect of identical CHOs mixtures against some other species was symptomatic. For example, growth inhibition of blueberry blossom blight causative agent, *B. cinerea*, was registered under significantly lower concentrations of CHOs, than both initial chitosans (Table 4). Enzymatic cleavage products of chitosan DD 50% demonstrated increment of growth-inhibiting activity against *A. alternaria* and *B. sorokiniana* contrary to *F. culmorum* that was suppressed in greater degree by CHOs prepared by hydrolysis of chitosan DD 85%.

Table 4
Minimal inhibiting concentrations of polymeric chitosans (DD 85 and 50%) and their enzyme hydrolysates[A] (including total CHOs mixtures) against several strains of phytopathogenic fungi and opportunistic bacteria

Fungal strains	**MIC, μg/mL**			
	Chitosan DD 85%	**CHOs DD 85%**	**Chitosan DD 50%**	**CHOs DD 50%**
Alternaria alternata (Fr.) Keisl. F-3047	70 ± 9	70 ± 5	80 ± 3	70 ± 3
Bipolaris sorokiniana (Sacc.) Shoemaker IB-G12	110 ± 8	100 ± 5	180 ± 5	120 ± 3
Botrytis cinerea Pers. F-894	190 ± 12	70 ± 4	210 ± 13	140 ± 12
Fusarium culmorum (W.G.Sm.) Sacc. F-844	110 ± 7	80 ± 7	80 ± 2	90 ± 5
Fusarium oxysporum Schlecht. emend. Snyder & Hansen F-137	110 ± 8	130 ± 14	100 ± 5	130 ± 10
Rhizoctonia solani J.G. Kühn F-895	20 ± 5	30 ± 6	30 ± 7	40 ± 6
Bacterial strains *Bacillus cereus* B-688	1100 ± 100	1610 ± 150	550 ± 30	500 ± 45
Enterobacter cloaceae IB-34-4CPA	90 ± 7	60 ± 3	ND[B]	ND[B]
Escherichia coli IBG-9	120 ± 15	70 ± 8	300 ± 25	140 ± 20
Pseudomonas aeruginosa IB-39D	1260 ± 110	> 2000	1390 ± 150	1170 ± 90

[A] The hydrolysates were prepared after 60-min treatment of original polysaccharides by the crude chitosanase from *B. thuringiensis* B-387.

[B] ND – not determined.

Comparison of antibacterial effects of enzymatic hydrolysates and original chitosans demonstrated evident activation of CHOs inhibition degree against *E. cloaceae* and *E. coli* (Table 4). By contrast, in the experiment, *B. cereus* and *P. aeruginosa* displayed significant decrease both to chitosans and CHOs; and activities of hydrolysates generally dropped relative to initial polymers (Table 4).

Discussion

The capability of *B. thuringiensis* B-387 to constitutive production of extracellular chitosanase was found for the first time in case of this species. Previously, such feature was demonstrated just in some strains of *Bacillus* spp. (Choi et al. 2004; Kim et al. 2004). Most of reported chitosanase-producing strains of

Bacillus, including *B. thuringiensis* belonging to *B. cereus* group, together with other known bacterial producers, require chitosan addition into culture medium (Koboyashi et al. 2011; Thadathil, Velappan 2014; Olicón-Hernández et al. 2016). Lee et al. (2007) characterizing expression of chitosanases family GH8 in variety of *B. thuringiensis* strains found chitosanolytic activity among 12 from 29 serovars of this species at their growth on LB medium, supplemented with soluble chitosan. Although, the strain Bt B-387 demonstrated sustainable chitosanase secretion in average range of 3–4 U/mL its productivity can be increased to the enzyme yield 12-12.5 U/mL through simple empirical fit of cultivation conditions without application of specific substrate induction and chitin-containing carbon sources (unpublished data). It is highly likely that further statistical optimization of crucial growth parameters will allow reach higher the chitosanase production by the Bt B-387 comparable or superior to efficiency reported for some other natural *Bacillus* strains (Choi et al. 2004; Chang et al. 2007; Gao et al. 2008). As distinct from the chitosanase, the chitinase of the Bt B-387 is inducible enzyme and it is found in minor quantities (≤ 0.1 U/mL) under cultivation of this strain in LB broth. Colloidal chitin as main carbon source induced the increase of chitinase production by the strain to approximately 0.3–0.7 U/mL depending on the substrate content (data are not presented). Differential expression of the chitosanase and chitinase in Bt B-387 is probably associated with specific mechanism of its insect pathogenesis that should be clarified in further.

The enzyme purification based on ultrafiltration, affinity sorption and cation-exchange chromatography steps detected sole isoform of the chitosanase M_w 40 kDa according to SDS-PAGE. Interestingly, the chitosanase fractions differencing in colloidal chitosan (DD 85%) sorption abilities demonstrated identical molecular mass values after final purification under same conditions (Fig. 2b). Most likely, this fact evidences on incomplete adsorption of the same chitosanase on the specific substrate, since it exerted similar physic-chemical and kinetic properties (data are not shown). Cation-exchange chromatography on CM-Sepharose was most effective and highly selective technique for the chitosanase purification to electrophoretically homogenous state. Kobayashi et al. (2011) purified earlier the chitosanase from *B. thuringiensis* JAM-GG01 with analogous manner using cation-exchange chromatography on CM-Toyopearl under alkaline conditions of borate buffer (pH 9.5) eluting the enzyme by linear gradient of 0.15 M NaCl. However, general purification scheme reported by these authors was more complex and included the steps of the enzyme re-chromatography on same resin at weakly acid equilibrating MOPS buffer (pH 6) and preliminary anion-exchange chromatography on SuperQ-Toyopearl. Molecular mass of the purified chitosanase (40 kDa) also indicated near value to the aforementioned chitosanase (43 kDa) from the *B. thuringiensis* JAM-GG01 (Kobayashi et al. 2011). Moreover, various characterized before chitosanases from *Bacillus* species belonging to glycosyl hydrolases (GH) family 8 demonstrated similar Mw values (40–46 kDa) (Mitsutomi et al. 1998; Kurakake et al. 2000; Choi et al. 2004; Gao et al. 2008; Liang et al. 2012). These are commonly bifunctional enzymes or chitosanases that capable to hydrolyze CM-cellulose and other glucans. Interestingly, GH 46 chitosanases displaying narrow substrate specificity commonly have the molecular masses in range 27–31 kDa (Kilani-Feki et al. 2013; Tomita et al. 2013; Luo et al. 2020). GH8-chitosanases are not infrequently reported in *B. thuringiensis* and related species (Lee et al. 2007; Gao et al. 2008). However, the chitosanase of Bt B-387

no manifested hydrolyzing activity against chitin, CM-cellulose and some other β-1,4 – and β-1,3-glucans (Table 3). Chitosanolytic activity of the purified enzyme markedly decreased against less acetylated chitosan form (DD 50%) suggesting its preference affinity and specific recognition to GlcN residues. The GH8 chitosanase from *B. thuringiensis* JAM-GG01 also not hydrolyzed cellulose and other glucans except for lichenan (Kobayshi et al. 2011). Biochemical and catalytic characteristics of the purified chitosanase generally are not exclusive among most of reported bacterial chitosanases, nevertheless, it is characterized with one of lowest K_M and higher specific V_{max} values. The temperature 70°C is crucial for the purified chitosanase of Bt B-387 which lost > 90% of its activity after 1 h of incubation. But most likely chitosan increases of the enzyme thermostability as it was reported for many bacterial chitosanases (Thadathil, Velappan 2014). Indirectly this fact is supported with retaining of obvious chitosan hydrolysis by the purified enzyme at 75–80°C. In spite of proximity of temperature optimum of the chitosanase from Bt B-387 to such of *B. thuringiensis* JAM-GG01 it markedly differs on its pH-optimum and demonstrates much higher thermostability (Kobayshi et al. 2011). The effect of tested metal cations and surfactants on Bt B-387 chitosanase activity is roughly the same that was reported begore for various bacterial chitosanases. Chitosan (DD 85%) hydrolysis by the chitosanase resulted to rapid formation of CHOs with DP ≥ 3–7 on the initial reaction step (5–10 min) in accordance with endo-mechanism. Longer-term incubation accumulated chitobiose, chitotriose and chitotetraose as main final products. It was coherent with almost complete conversion (95%) of chitosan by the crude enzyme complex of Bt B-387 into small water-soluble chitooligosaccharides molecules for 60-min reaction. Lower enzyme-substrate ratios increased the yield of low-molecular weight chitosans with M_w in range 14–46 kDa (Fig. 5). As known, LMWCs (M_w ≥15 kDa) demonstrate higher antimicrobial activity compared to smaller oligomers and CHOs with DP 2–6 (Park et al. 2008; Park et al. 2015; Zhao 2019). Lin et al. (2009) using various statistical models illustrated significant reverse relationship between logarithm of M_w and minimal inhibition concentration of LMWCs prepared from chitosans with DD 80 and 92% using chitinase, lysozyme and cellulase. Antibacterial activity of tested LMWCs increased within M_w ranges 8.5–13.5, 12-24.8, < 6-9.6 and 10.1–14.8 kDa (Lin et al. 2009). According to other data, antifungal activity of LMWCs with M_w 15–40 kDa sharply decreased compared to chitooligomers with M_w 4–10 kDa (Stepnova et al. 2007). Antimicrobial tests of CHOs mixtures resulted from extensive hydrolysis of 1% chitosans of DD 85 and 50% by crude chitosanase complex of Bt B-387 demonstrated ambiguous results manifesting both in decline and increase of growth-inhibiting effect against several fungal and bacterial strains as compared to initial polymers. This fact obviously is contingent on significant inter- and intraspecific variation of bacterial / fungal sensitivity to LMWC action determined by strain specific (Stepnova et al. 2007; Lin et al. 2009; Palma-Guerrero et al. 2010). Presumptive mechanisms for such specific as a rule are attributed to variety of interactions of LMWC with microbial plasma membrane lipids (Stepnova et al. 2007; Palma-Guerrero et al. 2010; Sanchez et al. 2017). Different sensitivity of bacterial and fungal cells to chitosan and CHOs is probably determined also with significant variations in hydrophobicity of cell surface for several species (Polyudova et al. 2019). In gram-positive bacteria, such as *Staphylococcus aureus*, development of resistance to chitosan probably correlates with acquired changes in its cell wall structure such as decrease of total negative charge of the cell surface, emergence of cross-resistance to some

antimicrobial agents, transformations in cell trophism and metabolism, receptor histidine kinase repair etc. (Raafat et al. 2017).

Our findings acknowledged retaining of acceptable antimicrobial activity for extensively hydrolyzed chitosan oligomers mixtures containing predominantly highly soluble small CHOs molecules. Alongside this, the control of such parameters as enzyme – substrate ratio and reaction temperature allow to shift the balance between hydrolyzing products upwards for LMWC with $M_{w(A)}$ 14–16 kDa regulating in so doing final antimicrobial activity of oligomers mixture. Although enzymatic conversion of high molecular chitosan to LMCW with wide range of Mw_A is considered sometimes as versatile problem realized only by coordinated application of several hydrolases (including non-specific enzymes) (Lin et al. 2009), its implementation through controlled destruction by alone enzyme or enzyme complex such as Bt B-387 chitosanase may by quite realistic. So, main results of this study conclusively illustrate versatile potential of the chitosanase from Bt B-387 for enzymatic preparation of water-soluble LMWC and CHOs exerting antimicrobial activity. More detail exploration for various controlled parameters contribution in this process is supposed to be done in further.

Summarizing, high basal level of the endochitosanase synthesis (3-3.5 U/mL) by *B. thuringiensis*, which does not require the addition of chitosan into nutrient medium as well as sizeable production potential of this culture allow to consider its as promising microbial producer of chitosanase for various biotechnological processes. The chitinase of *B. thuringiensis* B-387, unlike with chitosanase, is inducible enzyme secreted notably only in presence of colloidal chitin, therefore crude chitosanase preparation comprises just traces of chitinolytic activity. The purified enzyme differs in some their biochemical characteristics from previously reported Bt chitosanases. The chitosanase may effectively employed for the polymer conversion both into short CHOs and water-soluble LMWC. In the latter case, generation of bioactive LMWC with desired molecular weight is well-controlled by such parameters as enzyme / substrate ratio and incubation temperature. Enzymatic hydrolysates of chitosans with DD 85% and 50% demonstrate higher growth-inhibiting activity compared with initial polymers against certain plant pathogenic fungi and opportunistic pathogenic bacteria. The findings confirm the perspective of this enzyme for managed preparation of bioactive chitosan oligomers.

Declarations

Availability of data and materials All data generated or analyzed during this study are included in this published article and its supplementary information file.

Author contribution Main conceptualization and supervision by G. Aktuganov and A. Melentiev.; G. Aktuganov and V. Safina carried out researches, analyzed of data and prepared the paper. N. Galimzianova., E. Gilvanova, L. Kuzmina, Andr. Baymiev and S. Lopatin contributed with experiments, methodologies, and resources. G. Aktuganov, V. Safina and N. Galimzianova are responsible for discussion and editing.

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Conflict of Interest All the authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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Figures

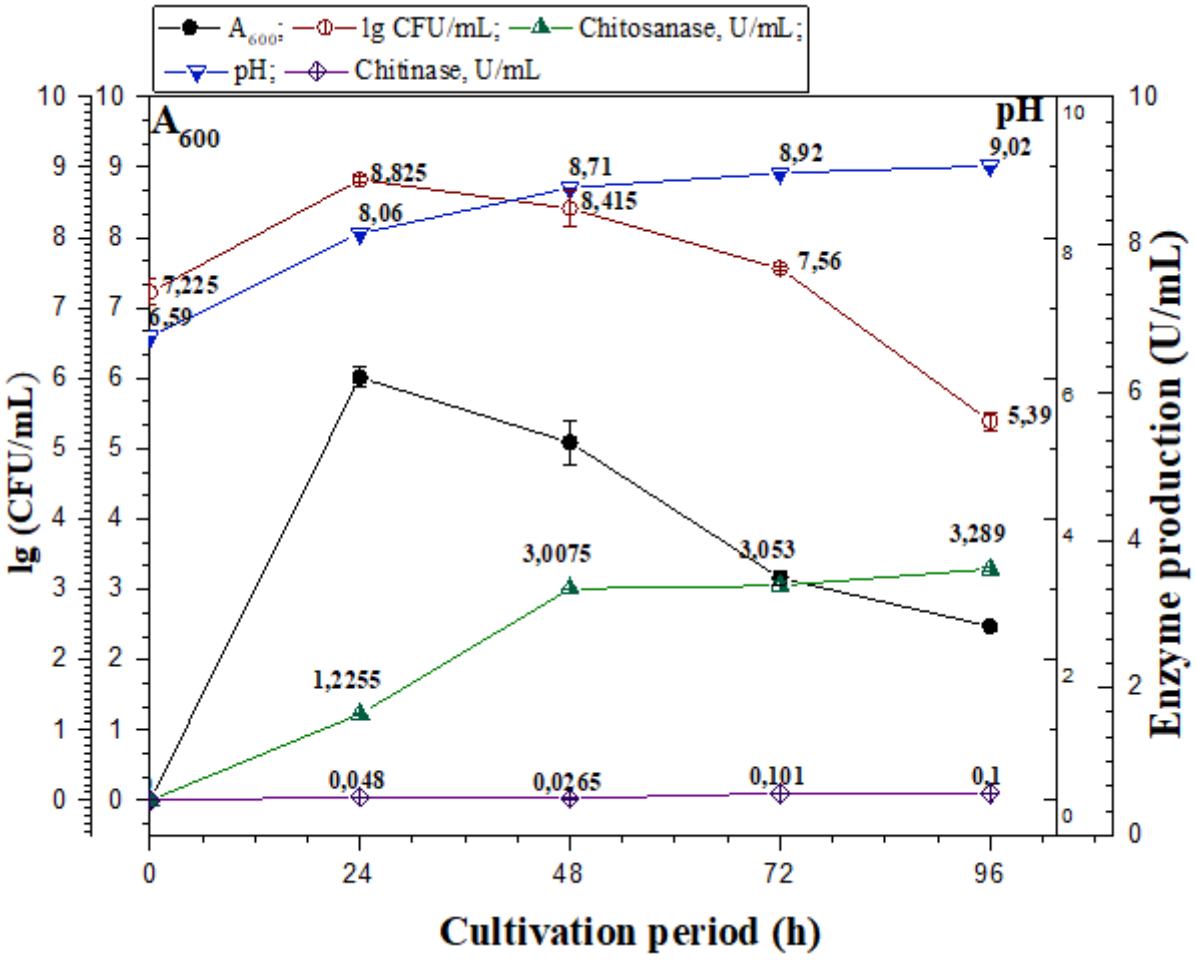

Figure 1

Growth rates of *B. thuringiensis* B-387, changes in its chitosanase / chitinase production and the culture pH dynamics during submerged cultivation in LB broth (36°C, 220 rpm).

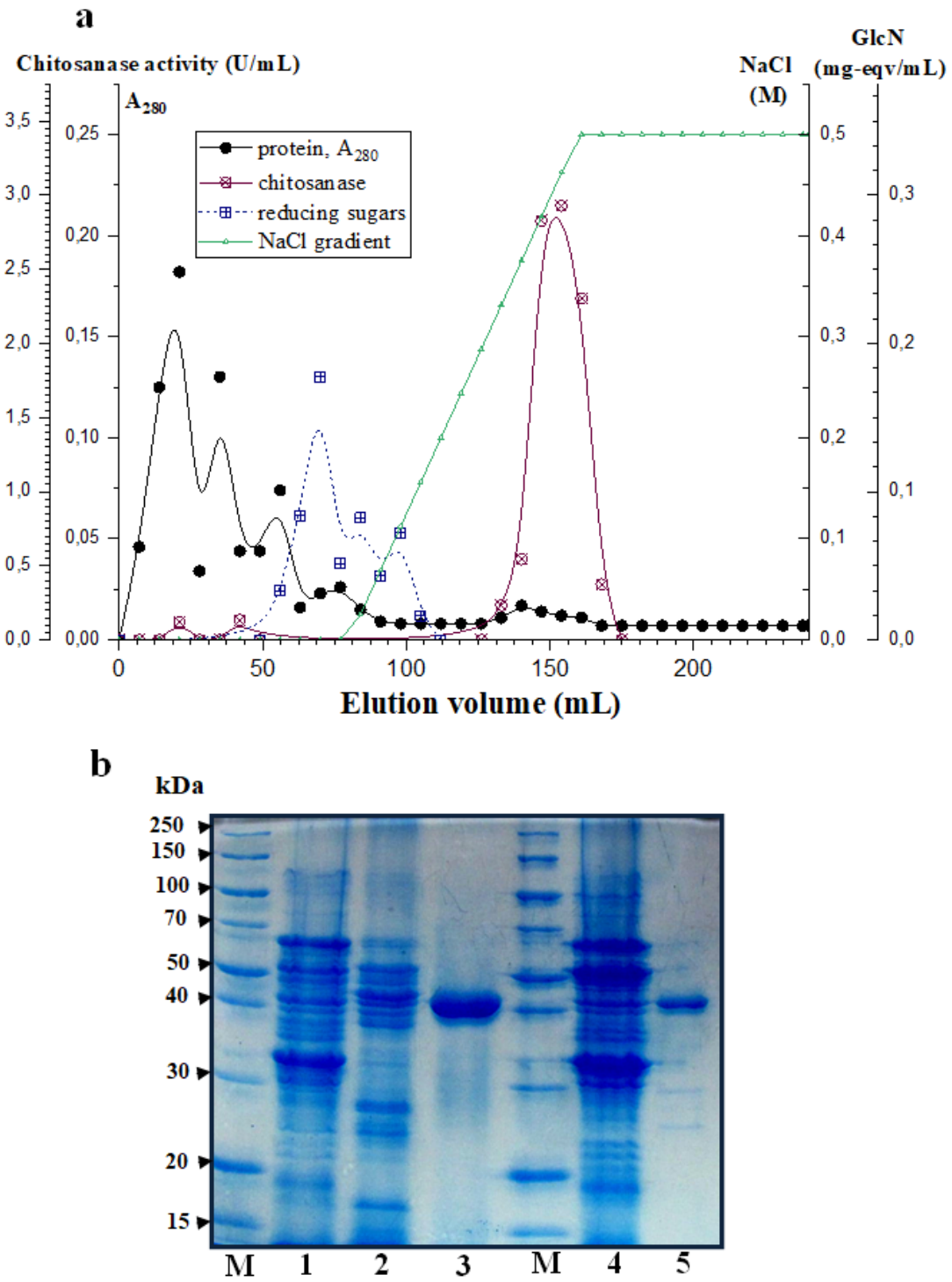

Figure 2

Purification of the chitosanase from *B. thuringiensis* B-387 by cation-exchanged chromatography on CM-Sepharose (a) and SDS-PAGE of the enzyme preparation under several purification steps (b). Reducing sugars presented in chromatography elution profile (a) are chitosan cleavage products released by adsorbed enzyme later on affinity sorption purification step. Lanes (b): 1 – crude culture supernatant; 2 – chitosanase fraction adsorbed by colloidal chitosan; 3 – chitosanase after final purification on CM-

Sepharose; 4 – fraction of proteins with residual chitosanase activity (>30%) non-adsorbed by colloidal chitosan; 5 – chitosanase from affinity non-adsorbed fraction (lane 4) purified on CM-Sepharose. M – standard marker proteins PageRuler Broad Range.

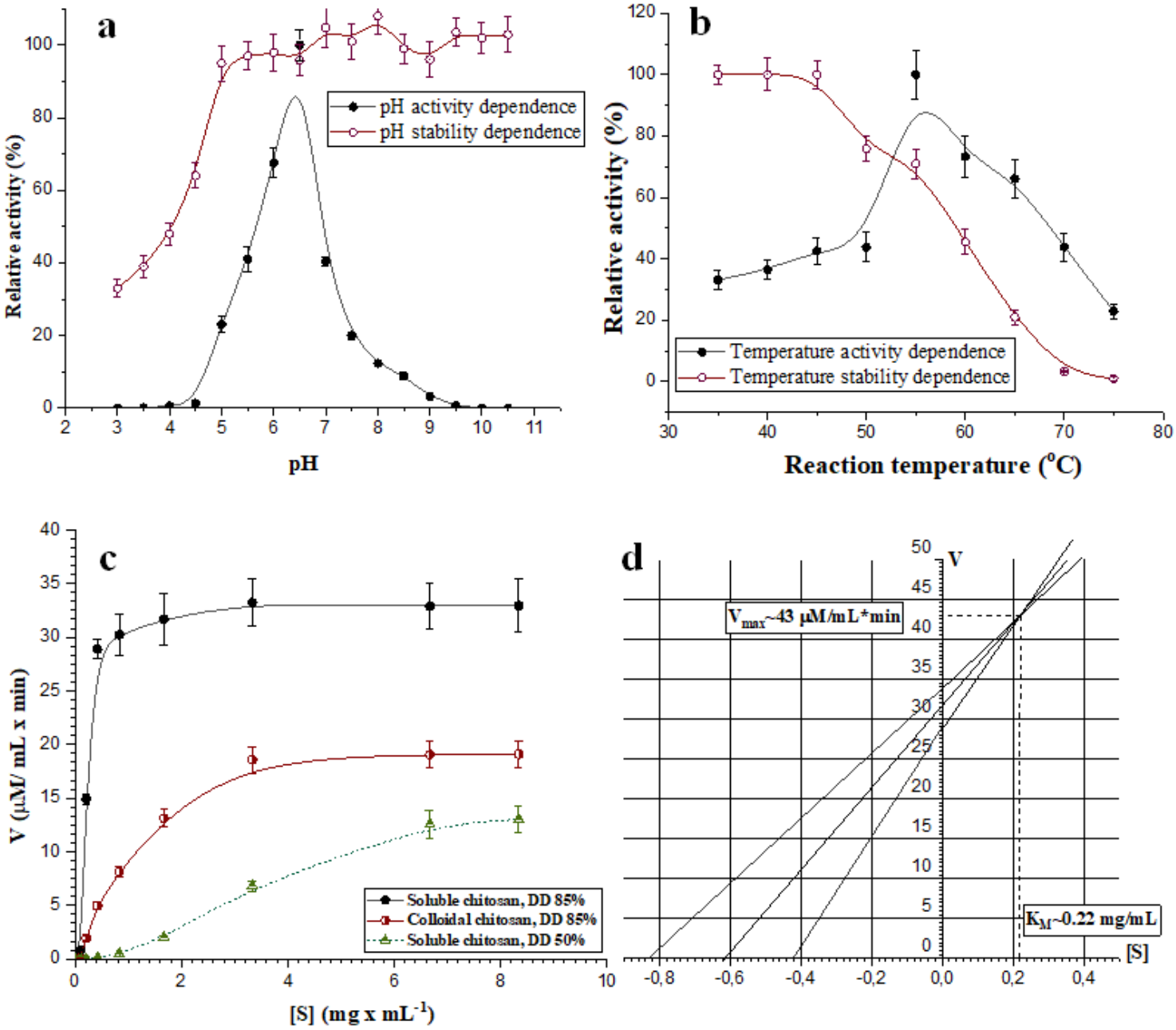

Figure 3

Physic-chemical (a, b) and catalytic (c, d) properties of the chitosanase from *B. thuringiensis* B-387. Effect of pH (a) and temperature (b) on the enzyme activity and stability. pH- and temperature stabilities were estimated by standard assay as residual activity of the enzyme after its 4 h and 1 h pre-incubation under several pH (5°C) and temperatures (pH 6), respectively, without chitosan addition. Substrate

concentration dependence curves for hydrolysis velocity of various chitosan forms by the chitosanase (c) and Eisental & Cornish-Bowden linear graphic of this dependence for soluble chitosan (DD 85%) (d).

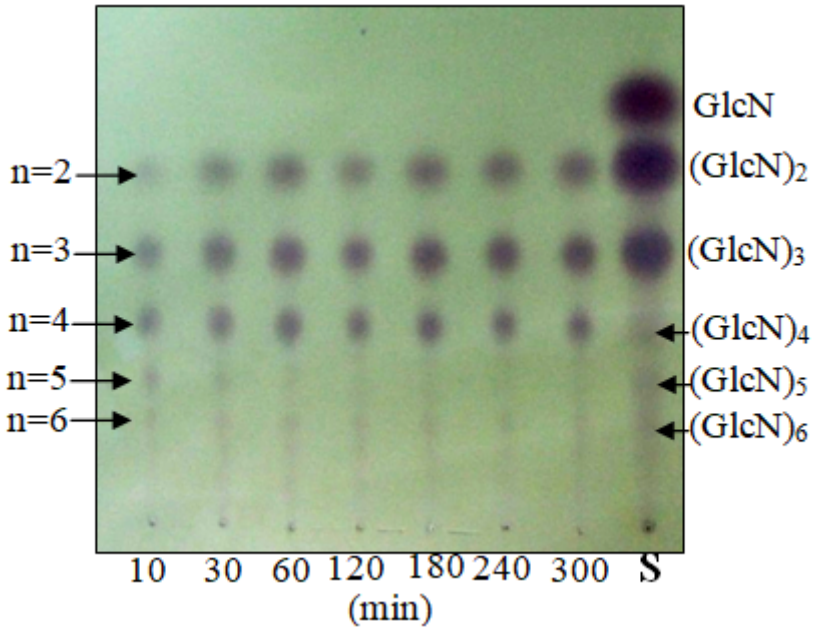

Figure 4

Thin-layer chromatography assay of chitosan (DD 85%, .5 mg/mL) hydrolysis products by purified chitosanase from *B. thuringiensis* B-387. S – standard (0.5 mM solution of D-glucosamine; n 2-5 – depolymerization degree (DP) of detected CHOs. Hydrolytic reaction was performed at pH 6 and 50°C under enzyme – substrate ratio 0.4 U/mg.

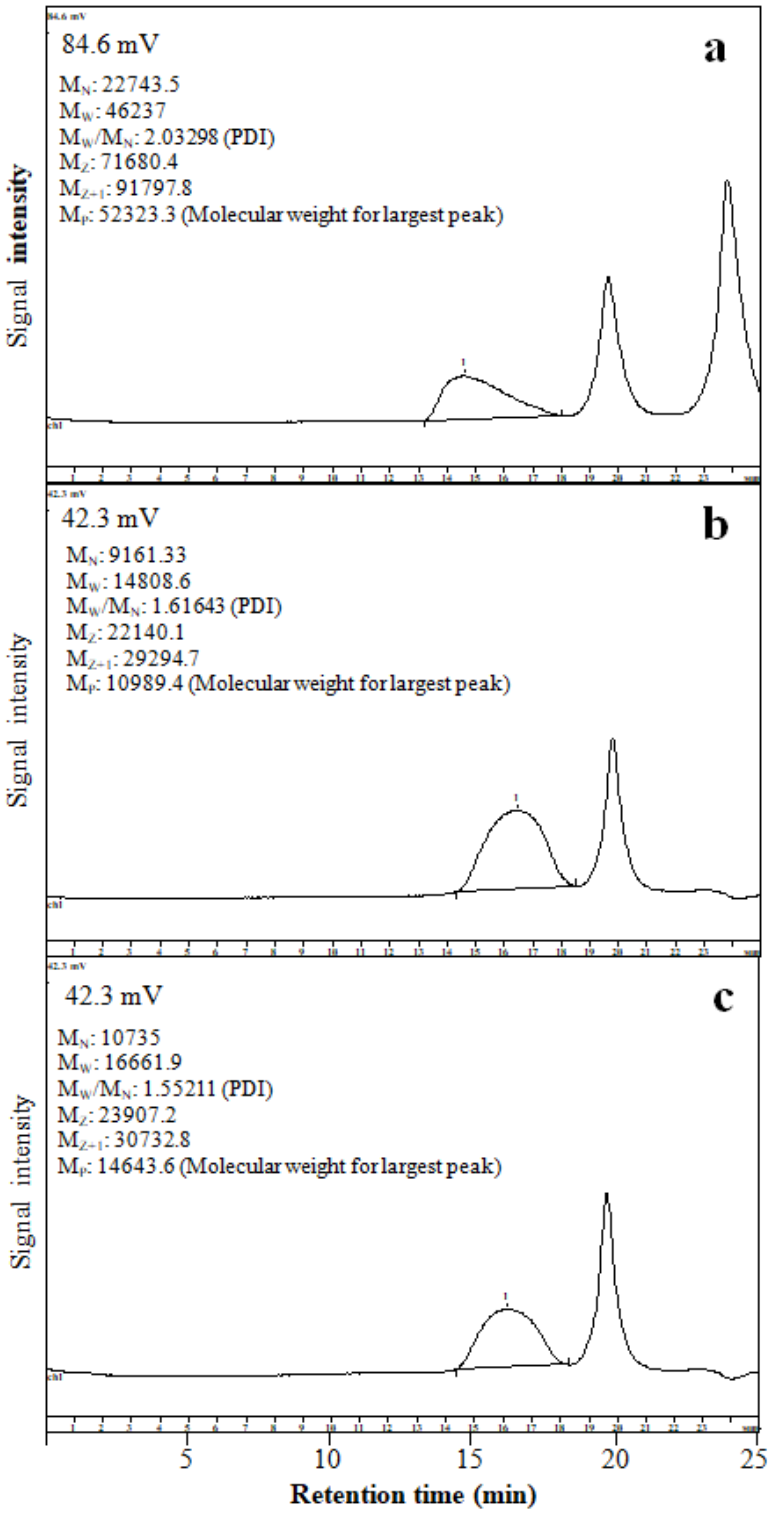

Figure 5

HP-SEC analysis of LMWCs prepared from chitosan (DD 85%) 60-min hydrolysis by the crude chitosanase under enzyme-substrate ratios (U/mg) 0.001 (a), 0.005 (b) and 0.01 (c). The incubation temperature for a and c was 50°C; for b - 70°C. PolySep-GFC-P 4000 column (7.8×300 mm). Injection volume was 20 µl.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Graphicalabstract.doc
- SupplementaryMaterial.doc