Purification, characterization, and action mode of a chitosanase from *Streptomyces roseolus* induced by chitin

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ABSTRACT

Chitosanase (EC3.2.1.132) catalyzes the hydrolysis of β-1,4-glycosidic bonds in chitosan, converting it into chitooligosaccharides, which exhibit versatile application potentials in food, pharmaceutical, and agricultural areas. In this paper we present a new inducible chitosanase, isolated, and purified from a bacterial culture medium of *Streptomyces roseolus* DH by precipitation with ammonium sulfate and combined column chromatographies. The SDS–PAGE results show its molecular mass is around 41 kDa, with a purity of more than 95%. The purified chitosanase exhibits optimum activity at 50 °C, pH 5.0. It is stable between 30 and 60 °C and at pH values between 5 and 7. It shows the highest activity towards colloidal chitosan and breaks down glycol chitosan and glycol chitin weakly. The enzyme is significantly inhibited by Cu^{2+}, Co^{2+}, Mn^{2+}, Zn^{2+}, and EDTA, but slightly activated by Mg^{2+}. Further action mode analysis based on chitosan oligomers and a polymer reveals that the chitosanase could split chitooligosaccharides with degree of polymerization (DP) >4 and chitosan in an endolytic manner. The resultant hydrolytes are mainly chitotrisaccharides, indicating it is suitable for the uniform bioconversion of chitosan and its derivatives with high efficiency.

1. Introduction

Chitosanase (EC3.2.1.132) is a glycoside hydrolase that acts on chitosan, a polymer mainly composed of β-1,4-linked D-glucosamine units. It usually plays an important role in the carbon and nitrogen cycles of the bacteria and fungi found extensively in soils.[1,2] A further potential use is for processing large amounts of chitosan and its derivatives, 60–80% of which are made through processing aquatic waste like shrimp and crab shells.[3] Compared to other chemical methods, whose product profiles are not only varied, but also unstable, enzymatic conversion by chitosanase produces size-specific chitooligosaccharides with particular physiological functions.[4–8]

Chitosanases are produced by organisms such as bacteria, fungi, and plants, and are speculated to fulfill roles in carbohydrate utilization, structural decomposition, and biological defense, respectively.[1,9–11] Based on the amino acid sequence similarities, these chitosanases are classified into various glycosyl hydrolase (GH) families such as GH-5, -7, -8, -46, -75, and -80, according to the carbohydrate-active enzymes database (http://www.cazy.org). Among them, prokaryotic chitosanases have received special attention, because they are naturally involved in maintaining the ecological balance of carbon and nitrogen cycles, especially in soils. For instance, two bacterial chitosanases from *Streptomyces* and *Bacillus* species have been well studied.[12,13] They belong to GH-46 or -8, with different conserved motifs that carry out enzymatic reactions through different mechanisms.[14–16] To date, there are no robust enzymatic methods for the degradation of chitosan that could provide an alternative to traditional chemical methods in an industrial scale. Furthermore, the molecular mechanisms of bacterial chitosanases have not, as yet, been fully elucidated due to the diversity of GH families and the different ways they degrade chitosan. Therefore, more information on chitosanase needs to be accumulated in order to allow a comprehensive understanding of their functions in nature. At present, wild-type chitosanases, which have been preserved through evolutionary selection, appear to be superior to genetically engineered enzymes.[10,14,16] Consequently, it is still practical to identify chitosanases with high activity from saprophytic microorganisms, for example *Streptomyces*, the largest genus of Actinobacteria in bacteria.

This paper describes the identification of another novel chitosanase, co-induced with the previously obtained chitinase by chitin from a high-yielding strain in soil, *Streptomyces roseolus*.[17] The chitosanase was purified to homogeneity and characterized.

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We further attempted to elucidate the action mode of chitosanase towards oligomers and a polymer by identifying which oligosaccharides were produced during enzymatic hydrolysis. This bacterial chitosnase represents another molecule through which to examine the mechanisms of enzymatic action, thus providing further insight into the biodegradation of chitosan.

2. Results and discussion

2.1. Purification of chitosanase from *Streptomyces roseolus*

Our previous results showed *Strepromyces roseolus* can produce chitosanase when induced by chitosan.[17] Here, an extracellular chitosanase was obtained from minimal culture medium of *Strepromyces roseolus* by adding powdered chitin as the sole carbon source and inducer. The precipitates, concentrated from fermentation supernatants by 80% ammonium sulfate precipitation, were collected for purification and applied to an anion-exchange chromatography column. Unlike the chitinase acquired in our previous studies, which was eluted quickly through the column,[18] the chitosanases purified in this case were negatively charged proteins at pH 8.5, and were tightly absorbed. They were not released until a high ionic strength, and were identified only in the latter elution peak (Fig. 1a). The active fractions were concentrated by centrifugal ultra-filtration and then subjected to size-exclusion chromatography (Fig. 1b). The fractions with high chitosanase activity were collected. After a two-step chromatography combination, the target enzyme was purified to near homogeneity, with purity estimated to be more than 95%, by grayscale scanning on Bandscan 5.0. The molecular mass of the purified enzyme was confirmed to be around 41 kDa by SDS–PAGE (Fig. 2). Further analysis of the native protein using gel filtration revealed that the chitosanase was active in a monomer (data not shown). To date, most chitosanases are characterized by their molecular mass in the range of 10–100 kDa, but bacterial ones, especially *Streptomyces* species, are usually between 30 and 40 kDa.[1,19,20]

A summary of the chitosanase purification process is displayed in Table 1. The enzyme was purified 13-fold, with an overall yield of 30% and a specific activity of 217 U/mg. These results show this strain to be one of the most active chitosanolytic strains that have been isolated through extensive soil sample screens, including *Microbacterium* sp. OU01 (~1000 U/mg),[23] *B. cereus* D-11 (~350 U/mg),[21] *Streptomyces* sp. (60–110 U/mg),[12] *B. circulans* (40–50 U/mg).[22] We can therefore conclude that this strain is superior to many others in the production of chitosanase.
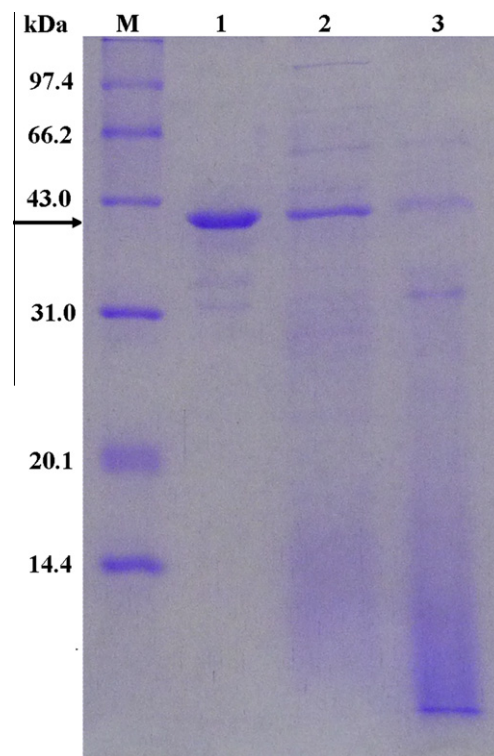

Figure 2. SDS–PAGE analysis of purification of chitosanase. Samples (~20 μg) were analyzed on 15% polyacrylamide gels and stained with Coomassie Brilliant Blue R-250. Lane M: standard protein marker; lane 1: chitosanase-active fractions collected from Superdex™ 75 column; lane 2: chitosanase-active fractions collected from Hiprep Q XL column; lane 3: ammonium sulfate precipitation. Arrow shows the purified chitosanase, with an estimated molecular mass of 41 kDa.

2.2. Effect of temperature and pH on chitosanase

The purified chitosanase from *S. roseolus* was active in the range 30–80 °C, being most active at 50 °C. It retained more than 80% of its highest activity at 60 °C (Fig. 3a). Furthermore, the thermal stability was investigated by measuring residual activity after pre-incubating the enzymes at different temperatures for 30 min. More than 90% of the initial activities were retained after incubation at 30, 40, 50, and 60 °C, which suggests enzymatic thermo-stability over a wide temperature range (Fig. 3a). This thermo-stability
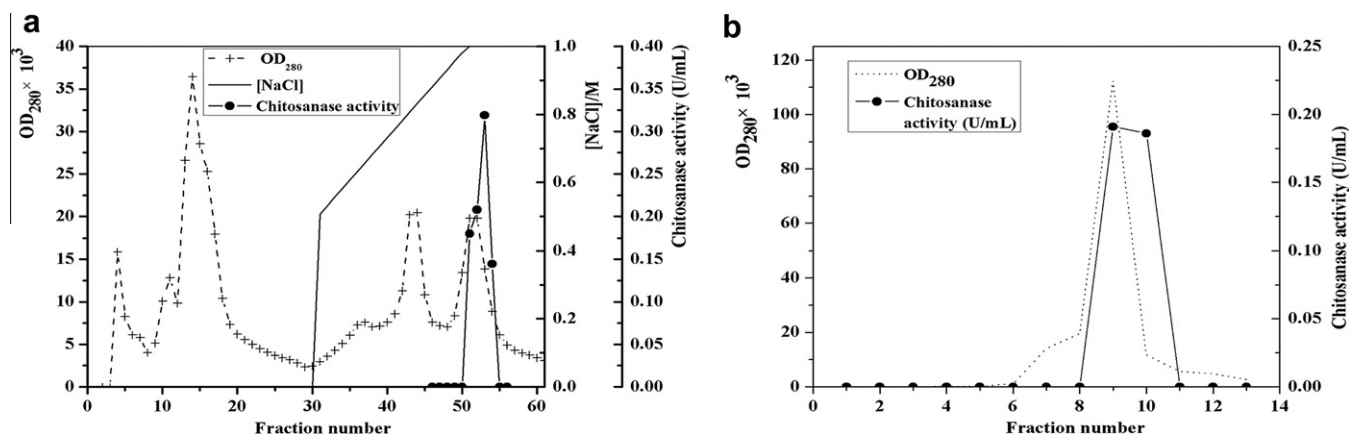

Figure 1. Purification of chitosanase from *Streptomyces roseolus* DH. (a) Elution profile of chitosanase on Hiprep Q XL column. (b) Elution profile of chitosanase on Superdex™ 75 column.

Table 1
Summary of purification of chitosanase from culture medium of *Streptomyces roseolus* DH

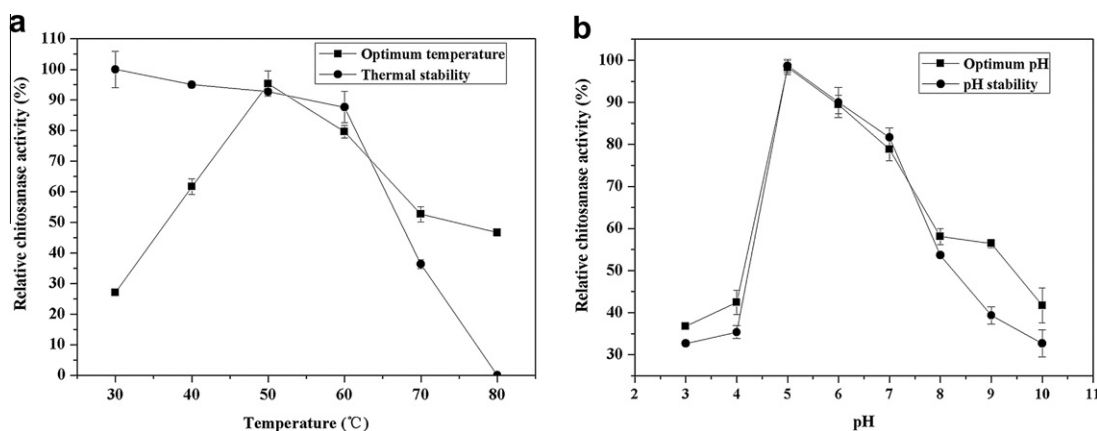| Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold	Yield (%)
Culture supernatant	43.5	720	17	1	100
(NH$_4$)$_2$SO$_4$ precipitation	28.1	503	18	1.1	70
HiPrep Q XL	6.7	404	60	3.5	56
Ultra-filtration	5.9	380	64	3.8	53
Superdex™ 75	1.0	217	217	13	30

Figure 3. Effects of temperature and pH on relative activity and stability of chitosanase from *Streptomyces roseolus* DH. (a) Optimum temperature (solid boxes) was measured under standard conditions, with the temperature range 30–80 °C being assessed. Thermo-stability (solid dots) was measured under standard conditions after pre-incubation of the enzyme at 30–80 °C for 30 min. (b) The optimum pH (solid boxes) was measured under standard conditions, with pH values varying from 3 to 10. The pH stability (solid dots) was measured under standard conditions after pre-incubating the enzymes in various buffers at 4 °C for 30 min.

makes it a better target for further investigation than most other chitosanses previously discovered.[9,19–21] For these reasons, it is considered a favorable choice for further applications, such as in the bioconversion of chitosan to oligomers.

The effects of pH on enzyme activity and stability were analyzed, and showed in Figure 3b. The optimum pH for chitosanase activity was identified to be pH 5.0, which agrees with the knowledge that most bacterial chitosanases display optimum activities at acidic pH values, especially from 4.5 to 6.5.[1,21] Compared to its inactivation at low pH values, the enzyme was relatively stable at pH 5–7, retaining around 80% of initial activity. The enzyme became more sensitive to pH changes over pH 7. Since the pH-dependent activity profile and pH-stability profile overlap with each other, the decrease of activity in low pH range may be due to the instability of the protein, rather than an acid–base catalytic mechanism reported in previous results.[12,15]

2.3. Effects of substrates and ions on chitosanase activity

Chitosanase activity was tested with five substrates: carboxylmethyl cellulose (CMC), colloidal/glycol chitosan, and colloidal/glycol chitin. The highest activity found was in the presence of colloidal chitosan (Table 2). It could not hydrolyze colloidal chitin or CMC, but decomposed glycol chitosan and glycol chitin at 12% and 11% of the activity of colloidal chitosan, respectively. Most

bacterial chitosanases hydrolyze chitosan efficiently, and chitin to a small extent. This is probably due to evolutionary adaption, both to the available natural substrates encountered in their environments and to different key functions within their hosts. However, fungal chitosanases have shown no detectable activity towards chitinous substrates.[20–23]

As shown in Table 3, the purified chitosanase from *S. roseolus* was significantly inhibited by the presence of Cu^{2+}, Co^{2+}, Mn^{2+}, Zn^{2+}, or EDTA. However, a slight activation was seen in the presence of Mg^{2+}. Other ions (Ba^{2+} and Ca^{2+}) showed nearly no effect on relative enzymatic activity.

2.4. Action mode of chitosanase

To determine the hydrolytic ability and enzymatic mode of the purified chitosanase with various oligoglucosamine (DP = 2–6) substrates, products were analyzed using thin layer chromatography (TLC). When chitosanase was incubated with dimers, trimers, or tetramers for 1 h at 37 °C, no obvious detectable lysates were observed (Fig. 4a), indicating that the chitosanase could not hydrolyze chitosan oligomers less than four glucosamine (GlcN) residues

Table 2
Effect of substrate specificity on chitosanase activity

Substrate	Relative chitosanase activity (%)
Colloidal chitosan	100
Glycol chitosan	12
Colloidal chitin	0
Glycol chitin	11
CMC	0

Table 3
Effects of various chemicals on chitosanase activity

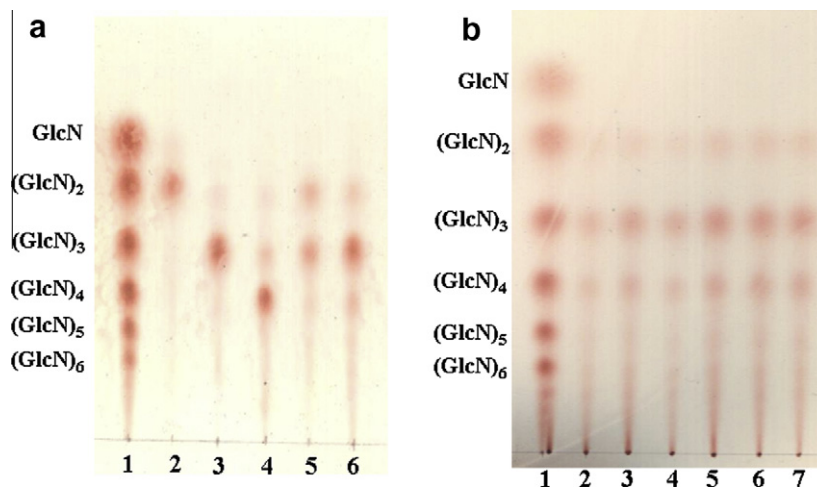| Chemicals (10 mM)	Relative chitosanase activity (%)
None	100
Cu^{2+}	27
Mn^{2+}	45
Ca^{2+}	98
Ba^{2+}	97
Mg^{2+}	103
Zn^{2+}	50
Co^{2+}	38
EDTA	68

Figure 4. TLC analysis of chitosanase-hydrolytic products. (a) Hydrolysates of chitooligosaccharides (DP = 2–6). Lane 1: standard glucosamine (GlcN) oligomers (DP = 1–6); lanes 2–6: enzymatic hydrolysates of $(GlcN)_2$, $(GlcN)_3$, $(GlcN)_4$, $(GlcN)_5$, and $(GlcN)_6$ after incubation at 37 °C for 1 h, respectively. (b) Hydrolysis of chitosan polymer. Lane 1: standard glucosamine oligomers (DP = 1–6). Lanes 2–3, 4–5, and 6–7: enzymatic hydrolysates of chitosan incubated at 37 °C for 0.5 h, 1 h, and 2 h, respectively.

long. As can be seen in Figure 4a, when pentamers were used as substrates, the $(GlcN)_5$ spot disappears, with $(GlcN)_2$ and $(GlcN)_3$ being produced at an approximate ratio of 1:1. This suggests that the chitosanase is active towards pentamers in an endolytic manner. When a hexamer $(GlcN)_6$ is used as a substrate a large amount of $(GlcN)_3$ was produced, along with smaller amounts of $(GlcN)_2$ and $(GlcN)_4$ (themselves at a ratio of about 1:1). This indicates that cleavage to $(GlcN)_3$ + $(GlcN)_3$ was more likely than cleavage to $(GlcN)_2$ + $(GlcN)_4$. Overall, the chitosanase from *S. roseolus* was able to hydrolyze chitopentose and chitohexose in endo-splitting modes, but it could not cleave the glycosidic bonds in sugar chains with DPs of less than four. These results are unanimous in bacterial chitosanases from the *Microbacterium* species.[23] They are also in agreement with results from previous studies using hexamers as substrates for chitosanases from *Streptomyces* sp. N174 and *Aspergillus* spp.[12,14,24]

Hydrolytic activity of chitosanase towards a natural chitosan polymer (more than 90% deacetylated) was investigated. TLC results show that most trimers occurred after 0.5 h of incubation, along with minor dimers and tetramers at a ratio of about 1:1, (Fig. 4b). During the hydrolytic reaction the amount of $(GlcN)_2$, $(GlcN)_3$, and $(GlcN)_4$ increased, but $(GlcN)_3$ remained predominant. This illustrates that the endo-type chitosanase can not only access the compact structure of chitosan, but also endo-cut the polymer into trimeric pieces efficiently and uniformly. However, it differs from the work using *Bacillus* sp. CK4 in which $(GlcN)_4$ was the major enzymatic product of chitosan.[9]

Taken together, these results demonstrate that the purified chitosanase can endo-cleave chitosanous substrates larger than tetramers, including a polymer. Product distribution was mainly chitotriose, with smaller amounts of chitobiose and chitotetraose being produced. Previously, it has been reported that the enzyme required substrates with three or more glucosamine residues.[1] In this study, we found that the enzyme could not only hydrolyze substrates with at least five glucosamine residues, but be able to access the polymer efficiently, producing predominantly trimeric mixtures from chitosan, indicating that it is fit for the rapid and uniform degradation of chitosan polymer.

3. Conclusion

A 41 kDa chitosanase has been purified from a culture medium of *Streptomyces roseolus* DH co-induced by chitin with chitinase,

and its properties and enzymatic mechanisms have been investigated. *S. roselous* has been identified as a high-yielding strain, and we observed good characteristics in purification parameters, including specific activity, purification fold, and yield. The optimum temperature for the enzyme was 50 °C and optimum pH was 5.0. It showed favorable thermo-stability between 30 and 60 °C and certain pH-stability in the range of pH 5–7. The purified chitosanase catalyzes an endo-type cleavage reaction in chitosan oligomers and a polymer efficiently, and liberates mainly chitotriose products. This indicates it could be beneficial for efficient and uniform bioconversion of chitosanous materials.

4. Experimental

4.1. Materials

Chitosan (more than 90% deacetylated) was purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Colloidal chitosan was prepared as described previously.[17] Chitosan dimers, trimers, tetramers, pentamers, and hexamers were obtained from Lianyungang Haikang Biological Technology (Jiangsu, China). Glycol chitin[25] was prepared from glycol chitosan (Sigma, USA). Columns of HiPrep 16/10 Q XL and Superdex™ 75 HR 10/30 were purchased from GE Healthcare. Amicon Ultra-4 (10 kDa) was from Millipore (USA). All other reagents used were of at least analytical grade.

4.2. Enzyme purification

The strain *Streptomyces roseolus* DH was originally isolated and screened from the shrimp shell-rich soils near East Sea in Shanghai (China). For the production of chitinase[18] and chitosanase, it was incubated in culture medium containing 1.0% chitin, 0.05% KH_2PO_4, 0.05% NaCl, 0.05% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, 0.4% NH_4NO_3, pH 7.0 on a rotary shaker at 28 °C for three days. The supernatants were precipitated and centrifugally collected at 80% saturation of ammonium sulfate precipitation. The precipitates were then dissolved in a small amount of 50 mM Tris–HCl buffer (pH 8.5) and dialyzed against the same buffer. The dialysates were then loaded onto a HiPrep Q XL 16/10 column, washed with the above buffer and eluted with a linear gradient of 0–1 M NaCl-containing buffer at a flow rate of 1 mL/min. Fractions of 2.5 mL were collected. The fractions with high chitosanase activities were

pooled and concentrated by ultra-filtration (Amicon Ultra-4). The concentrates were used for a further gel filtration step on a Super-dex™ 75 HR 10/30 column. Fractions of 1.5 mL were collected. The chitosanase-active fractions were obtained and combined for subsequent characterization.

Protein content was determined by Bradford assay[26] with bovine serum albumin as the standard. After column chromatography, the protein concentrations in collected fractions were spectrophotometrically estimated at 280 nm.

4.3. Enzyme assay

Chitosanase activity was measured with colloidal chitosan as a substrate. To 0.5 mL enzyme solution, 1.5 mL substrate solution, 0.3% colloidal chitosan in sodium acetate buffer (50 mM, pH 6.0), was added. The mixture was incubated at 50 °C for 30 min and the reaction terminated by being boiled at 100 °C for 5 min. After centrifugation at 10,000g for 2 min, the amount of reducing sugar produced in the supernatant was determined using a modified dinitrosalicyclic acid method. One unit of chitosanase activity was defined as the amount of enzyme that liberated reducing sugars corresponding to 1 μM glucosamine in 1 min.[27]

4.4. Electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to Laemmli,[28] using 15% (W/V) polyacrylamide resolving gels and 5% (W/V) polyacrylamide stacking gels.

4.5. Effects of temperature and pH on enzyme activity and stability

The optimum temperature for chitosanase activity was studied by incubating the purified enzyme with substrates at temperatures ranging from 30 °C to 80 °C under standard assay conditions. Thermal stability was determined by pre-incubating the enzyme, without substrate, in sodium acetate buffer (pH 6.0) for 30 min at temperatures from 30 °C to 80 °C. The residual activity was measured under standard conditions.

The optimum pH of chitosanase was investigated in the same way as enzyme assay described above, except that reaction buffers had pH values in the range 3–10, using 50 mM of each buffer: glycine–HCl (pH 3), acetate (pH 4–6), potassium phosphate (pH 7–8), glycine–NaOH (pH 9–10). To determine pH stability, the enzymes were pre-incubated at various pH values, without substrates, in the above buffer systems at 4 °C for 30 min and then subjected to enzyme assay under standard conditions. All measurements were repeated at least three times.

4.6. Effect of various chemicals and substrates on chitosanase activity

The effects of various chemicals on enzyme activities were investigated by adding different reagents, at concentrations of 10 mM, to reaction mixtures and pre-incubating the mixtures for 30 min at 4 °C. The following ions and compounds were used in the assay: Mn^{2+} ($MnCl_2$), Cu^{2+} ($CuSO_4$), Mg^{2+} ($MgCl_2$), Ba^{2+} ($BaCl_2$), Co^{2+} ($CoCl_2$), Ca^{2+} ($CaCl_2$), Zn^{2+} ($ZnSO_4$), and EDTA. The residual activities were then measured under standard assay conditions and expressed with relative activity termed as percentage ratio of the specific activity of the enzyme with chemicals to that without chemicals.

Colloidal chitosan, glycol chitosan, and its analogues (colloidal chitin, glycol chitin, and carboxylmethyl cellulose) were used as substrates and the corresponding enzymatic activities were measured in the same way as the standard enzyme assay. The activity using colloidal chitosan as a substrate was set as 100%.

4.7. Mode of chitosanase action

Thin layer chromatography (TLC) was used to determine the enzymatic hydrolysis mode of chitosanase on chitosan oligomers and a polymer. Aliquots (1 μl) of the reaction mixtures were chromatographed on a Silica Gel 60 plates (Merck, Germany) with ethyl acetate–methanol–ammonia–water (5:9:1:1.5, v/v). Products were detected by rinsing the plates with p-anisaldehyde–ethanol–sulfuric acid–acetate reagent (1:18:1:0.2, v/v) and baking at 120 °C for 5 min.[29]

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