Purification, characterization and cloning of a chitinase from *Stenotrophomonas rhizophila* G22

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Abstract

In the presented research the extracellular chitinase of *Stenotrophomonas rhizophila* G22 was biochemically and molecularly characterized. The studied enzyme was purified from a 72-h bacterial culture about 14 times, with a recovery of 63%. The molecular weight of the purified protein was estimated at 50 kDa by SDS-PAGE. The enzyme showed high activity against colloidal chitin. Significantly lower activities were observed with native chitin powder and chitosan. Adsorption of the enzyme to colloidal chitin and to powdered chitin at the level of 75% and 37%, respectively, was observed after 30 min of reaction. Optimum temperature and pH were 37 °C and 5.9, respectively. The enzyme demonstrated higher activity against nitrophenyl- β -D-N, N', N''-triacetylchitotriose and approx. 5 times lower activity for 4-nitrophenyl-N, N'-diacetyl- β -D-chitobiose. The enzyme is an endochitinase, which is confirmed by the K_m and V_{max} values determined in the studies. *S. rhizophila* G22 endochitinase was inhibited in the presence of cysteine-specific inhibitors, which indicates the role of cysteine moieties in the mechanism of catalysis or in stabilisation of the enzyme molecule. Also Ca^{2+} and Mn^{2+} ions may stabilise the protein's spatial structure. SDS and ions: Fe^{2+} , Cu^{2+} , Co^{2+} , Zn^{2+} inhibited the activity of enzyme. A full-length (2109 bp) gene coding chitinase from *S. rhizophila* G22 was obtained. Four domains typical for glycoside hydrolase family 18 (GH 18) chitinases were identified: catalytic Gly_18, chitin-binding—ChtBD3, type-III fibronectin—FN3 and polycystic kidney disease domain—PKD domain.

Keywords Chitinase · *S. rhizophila* · Gene sequence · Family GH18

Introduction

Chitinases are enzymes decomposing chitin—one of the most abundant natural polymers. This mechanically strong polysaccharide is a building material of the exoskeletons of insects, crustaceans, molluscs, some nematodes and is also a component of the cellular walls of fungi, protozoans and algae (Swiontek Brzezinska et al. 2014). Chitin is composed of *N*-acetyl-D-glucosamine (GlcNAc) monomers linked together with β -1-4-glycosidic bonds.

Three polymorphic forms of chitin are known: α , β and γ . The most common α -chitin, found in, among others, shrimps, crabs, crayfish and lobsters, has antiparallel microfibrils of chitin bound with strong intermolecular hydrogen bonds. The polymorphic β -form, with parallel chitin chains, is found in squids and cuttlefish. β -Chitin is more reactive and better soluble. The third form of chitin, obtained from fungi and yeast— γ -chitin, is composed of a combination of parallel and antiparallel chains (Majeti and Ravi 2000; Kumirska et al. 2011).

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In ecosystems, chitin is recycled by enzymes. As a result of the process, carbon and nitrogen are returned to circulation (Vogan et al. 2008). Enzymatic decomposition of this recalcitrant polysaccharide is a multi-stage process. It is catalysed by chitinases [EC 3.2.1.14] and β -*N*-acetylhexosaminidases [EC 3.2.1.52] (and by recently reported lytic polysaccharide monooxygenases [EC 1.14.99.53]) (Kuusk et al. 2019). Considering similarities of their amino acid sequence, chitinases and β -*N*-acetylhexosaminidases are classified into four (18, 19, 23 and 48) and six (3, 5, 18, 20, 84 and 116) glycoside hydrolase (GH) families, respectively (<https://www.cazy.org/>). Depending on the localisation of bonds hydrolysed by chitinases, they are classified as endochitinases, which hydrolyse random β -1-4-glycosidic bonds inside the chitin chain and exochitinases, which cleave the disaccharide—chitobios from one of the chain ends (Davies and Henrissat 1995).

In a chitinase molecule several domains may be defined, the most important of which is the catalytic domain. Besides the catalytic domain there are also: a signal sequence, a chitin-binding domain, a type-III fibronectin domain, a polycystic kidney disease domain and serine or threonine-rich regions usually located between the other domains (Huang et al. 2012).

Chitinases are present in many organisms and play important roles. For instance, acidic mammalian chitinases (AMCase) are effectors of the immunological response and their increased activity is observed in people with asthma. People with deficiency of chitotriosidases demonstrate an increased rate of fungal colonisation (Adrangi and Faramarzi 2013; Bohr et al. 2015). Similarly, too low activity of β -*N*-acetylhexosaminidases causes human disease (Liu et al. 2018).

Chitin-degrading enzymes are also a powerful biotechnology tool, especially used as a combination of chitinases with different sources of origin and specificities. A powerful synergistic effect in the action of bacterial chitinases and insect *N*-acetyl-D-glucosaminidase was observed in the decomposition of natural chitin substrates (Zhu et al. 2016; Chu et al. 2019). Environmental chitinolytic bacteria may be potentially used as natural fungicides, applicable in biological protection of plants. The high chitinolytic activity of *Stenotrophomonas* genus bacteria indicates their potential role in biocontrol (Schmidt et al. 2012; Hernandez-Montiel et al. 2017). However, no detailed scientific report on *Stenotrophomonas rhizophila* chitinase has yet been published. Therefore, the aim of this study was to characterize the biochemical properties of the endochitinase synthesized by *Stenotrophomonas rhizophila* G22. The study yielded also the full nucleotide sequence of the chitinase-coding gene. The sequence was subsequently analysed for the presence of chitinase typical domains.

Materials and methods

Identification of bacteria

Chitinolytic *Stenotrophomonas rhizophila* G22 bacteria were isolated from the rhizospheric zone of spring barley. Species determination was based on morphological, biochemical characteristics and analysis of the 16S rRNA gene sequence. The Genomic Mini AX Bacteria (A&A Biotechnology, Poland) set was used for isolation of genomic DNA from G22 bacteria. Isolation effectiveness was verified by electrophoresis in 0.8% agarose gel. Isolated DNA was stored at -20°C . PCR was completed using universal starters: F27 and R1492. The obtained PCR product was sequenced. Resulting 16S rRNA gene sequence for G22 bacteria was deposited in the DDBJ/EMBL/GenBank with the number: LC494522.

Bacterial culture and detection of chitinolytic activity

Chitinolytic activity of studied bacteria was detected on solid medium composed of (g/l): colloidal chitin 10, yeast extract 2, soy peptone 2.5 and agar 18. After 5 days of cultivation at 28°C clearances of the medium were observed, resulting from chitin decomposition by the active enzyme.

To obtain chitinase for study, bacterial cultures were carried out in liquid media composed of (g/l): colloidal chitin 10, yeast extract 1, soy peptone 1.5. The medium pH was 6.5. Culture temperature was 28°C and shaking at 120 rpm was applied. The 72-h bacterial cultures were centrifuged ($13,000\times g$, 10 min), and the collected supernatant was used as a source of chitinase.

Preparation of colloidal chitin

Colloidal chitin was prepared using native chitin from shrimp shells (Sigma), according to the Roberts and Seli-trennikoff method (1988).

Determination of enzymatic activity

Chitinolytic-activity was determined spectrophotometrically, according to Miller's method (1959) with 3',5'-dinitrosalicylic acid (DNS). Colloidal chitin suspended in 100 mM acetate buffer pH 5.9 was used as a substrate, at final concentration in the reaction mixture of 50 mg/ml. Reactions were stopped by centrifugation of substrate and uptake of the supernatant. DNS was added to the supernatant (volume ratio supernatant: DNS 1:1) and the mixture was heated for 10 min at 100°C to determine the increase of

reducing sugars during the reaction. Absorbance of samples was measured at a wavelength of 540 nm. The amount of released reducing sugars was calculated using a standard curve for acetylglucosamine (GlcNAc). The activity unit was 1 μmol of GlcNAc obtained from enzymatic hydrolysis during 1 min using 1 ml of the enzyme.

Purification of chitinase

All stages of the enzyme purification were carried out at 4 °C. Bacterial cultures were centrifuged and proteins contained in the supernatant were fractionated with ammonium sulphate (0–30, 30–85% saturation). The sediment following centrifugation was dissolved in 20 mM Tris–HCl buffer, pH 6.8 and dialysed against the same buffer for 12 h. Dialysed-enzyme solution was applied on chromatographic bed of DEAE–Sephadex (Sigma). The chromatographic column was equilibrated with 20 mM Tris–HCl buffer, pH 6.8. Fractions demonstrating higher activity were concentrated on Vivaspin filters (cut-off 5 kDa). The obtained enzyme preparation was applied on the bed for molecular-sieve chromatography (Superdex 200, GE Healthcare). Active fractions were used for studies of the enzyme properties.

Electrophoretic separations and zymograms

For monitoring the degree of protein purification and estimation of molecular weight, electrophoretic separations were carried out under denaturing conditions (SDS–PAGE), in 4% stacking gel and in 12% separating polyacrylamide gel (Laemmli 1970). Protein bands were stained by Coomassie Brilliant Blue R-250. Native electrophoretic separations were carried out in 10% separating polyacrylamide gel with 0.1% chitin glycol to detect chitinase activity. After electrophoretic separation, the gels were incubated in 50 mM acetate buffer, pH 5.9 at 37 °C for 3 h, and subsequently stained in 0.1% Congo red aquatic solution.

Protein level determination

Protein content in samples was detected using the Bradford method (1976), with BSA solutions ranging from 0.1 to 1.4 mg/ml as a standard protein.

Determination of optimum temperature and pH of purified chitinase

Optimum conditions for activity of the studied enzyme were determined using colloidal chitin as a substrate. Optimum temperature for chitinase was determined at 20 to 55 °C, and optimum pH at 3.5–8.5. For this purpose acetate buffer (pH from 3.5 to 6.0) and Tris–HCl buffer (pH 6.5–8.5) were used.

Substrate-specificity of purified chitinase to synthetic and natural substrates and kinetic parameters

Synthetic substrates (Sigma, Chitinase kit): 4-nitrophenyl- β -D-*N,N',N''*-triacylchitotriose (specific for endochitinases), 4-nitrophenyl *N,N'*-diacetyl- β -D-chitobioside (for exochitinases), -Nitrophenyl *N*-acetyl- β -D-glucosaminide (for *N*-acetyl-glucosaminidase) were used for determination of specificity of the enzyme for glycosidic bond hydrolysed in the substrate molecule. In the reaction mixtures, substrates were present at the final concentration of 1.0 mM. The Michaelis–Menten kinetic parameters (K_m and V_{max}) were determined against synthetic substrates, *p*-nitrophenyl derivatives of *N*-acetylglucosamine at a concentration of 0.25 to 3 mM using Lineweaver–Burk plots.

The reaction was carried out for 20 min at 37 °C and was terminated with 200 mM sodium carbonate. The amount of *p*-nitrophenol released in the reaction was determined photometrically at a wavelength of 420 nm. One unit of chitinase activity (U) was defined as the amount of enzyme which yields 1 μmol of *p*-nitrophenol per hour under the standard assay conditions.

Substrate-specificity was determined with natural substrates: native chitin from shellfish shells, chitin glycol, glycol chitosan, chitosan, carboxymethylcellulose, laminarin and xylan. In the reaction mixtures, substrates were present at the final concentration of 20 mg/ml. Reductivity increase was determined using the Miller's method (1959) described above.

Chitinase binding to colloidal and powdered chitin

The chitinase *S. rhizophila* G22 binding rate to colloidal chitin and native chitin from shellfish shells was tested for 0–30 min at 15 °C. A reaction mixture containing a particular substrate (10 mg/ml), purified chitinase and 50 mM acetate buffer, pH 5.9, was prepared. Samples were taken every 5 min and the reaction was stopped by centrifugation of the substrate. The amount of unbound enzyme was determined in the supernatant using 4-nitrophenyl- β -D-*N,N',N''*-triacylchitotriose as a substrate.

The effect of metal ions and selected chemicals on activity of the chitinase

Metal ions (Mn^{2+} , Zn^{2+} , Fe^{3+} , Cu^{2+} , Ca^{2+} , Co^{2+} , Mg^{2+} , Ba^{2+}) with final concentration of 1 mM and 5 mM were used in the reaction mixture. Inhibitors or activators (phenylmethylsulfonyl fluoride, iodoacetamide, Tween 20, Triton x-100, urea, dimethyl sulfoxide, sodium lauryl sulphate and ethylenediaminetetraacetic acid) were used in different concentrations. Before determination of activity, the enzyme

was pre-incubated with metal ions or the above-mentioned chemicals for 30 min at 20 °C, and then incubated with the substrate (colloidal chitin). The activity of the enzyme was determined using the photometric method with DNS.

Amplification of the chitinase gene

Pairs of starters used in PCR:

1. F 5'TGACCCACATCAACTACTG, R 5'GTAGCCCATCTTCTCGGT3
2. F 5'GCTCAAGACCCTCAACTGG, R 5'GGTGGCCATGCAGTACAA
3. F 5'AGGACATCTGGAATGCCC, R 5'GCTGCTTGA GCTGGTTCC
4. F 5'GGATGAAGTAGACATGCCAGG, R 5'GTTGCG GTACACGTCGTAG

For amplification of the chitinase gene, DNA polymerase Pfu (EURX, Poland) and genomic DNA from G22 bacteria as a matrix for the reaction were used. Obtained PCR products were sequenced. Combination of nucleotide sequences resulted in a 2109 bp long gene. The gene sequence was deposited in the DDBJ/EMBL/GenBank with the number: LC497040.

All results presented in this paper in the form of numerical values were means from three independent determinations. The mean error, reflecting maximal deviation of the results of measurements from the mean, did not exceed 5%.

Results

Isolates of chitinolytic bacteria

Six chitinolytic bacterial strains were isolated from the rhizospheric zone of barley. The most active one was used in the study. The strain was identified as *Stenotrophomonas rhizophila*, designated in the laboratory as G22, 16S rRNA sequence access No.: LC494522. Sequence analysis of the 16S rRNA gene demonstrated a high similarity (98.6%) of the analysed strain to *Stenotrophomonas rhizophila* DSM

14405 (CP007597) and (98.5%) to *Stenotrophomonas rhizophila* strain GL5 (HF545317).

Purification of chitinase and electrophoresis

A high chitinolytic activity was observed already in 72-h cultures of *S. rhizophila* G22 in liquid media containing 1% colloidal chitin and 0.15% soy peptone and 0.1% yeast extract. After centrifugation (10,000×g, 10 min.) of the bacterial culture, a chitinolytically active supernatant was obtained (1200 ml). The supernatant was used for purification of the chitinase.

Purified chitinase from *S. rhizophila* G22 was obtained from a three-stage procedure: protein salting out with ammonium sulphate, ion-exchange chromatography and molecular filtration. The complete procedure yielded 13.8-times purified chitinase protein demonstrating specific activity of 2.34 U/mg of protein and activity recovery at 63% (Table 1). Homogeneity verification of purified protein and determination of its molecular weight were carried out with SDS-PAGE. The molecular weight of the purified protein was estimated at 50 kDa (Fig. 1). 2 µg of pure protein was applied to the gel. Zymograms for both non-purified and purified protein demonstrated that the studied G22 bacteria secreted a single isoform of chitinase active against chitin glycol (Fig. 2).

Optimal temperature and pH of chitinase

Optimum temperature for *S. rhizophila* chitinase was 37 °C. The enzyme demonstrated activity over the whole tested range of temperatures from 20 to 50 °C, but above the optimum temperature the activity was rapidly reduced (Fig. 3). Optimum pH for chitinase was 5.9. The enzyme demonstrated chitinolytic activity over broad pH range from 4.5 to 8 (Fig. 4).

Substrate-specificity and kinetic parameters of chitinase

The enzyme demonstrated higher activity to nitrophenyl-β D N,N',N''-triacylchitotriose, a substrate for endochitinases,

Table 1 Purification of chitinase from *S. rhizophila* G22

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification fold
Cell-free supernatant	151	904	0.17	100	1
Ammonium sulfate precipitation (30–85%)	133	529	0.25	88.1	1.5
Anion exchange chromatography DEAE Sepharose (0–0.5 M NaCl)	103	108	0.95	68.2	5.6
Gel-filtration chromatography, Superdex 200	96	41	2.34	63	13.8

Fig. 1 SDS-PAGE of the purified chitinase of *S. rhizophila* G22

Fig. 2 Zymograms of the non-purified chitinase (left) and the purified chitinase (right)

and several times lower to 4-nitrophenyl-*N*, *N'*-diacetyl- β -D-chitobiose, a substrate for exochitinases. Additionally, in the studies chitinase did not exhibit activity against 4-nitrophenyl-*N*-acetyl- β -D-glucosaminidase—a substance for *N*-acetyl-glucosaminidase. For -nitrophenyl- β D *N*, *N'*, *N'*-triacetylchitotriose, a lower K_m and higher V_{max} were also obtained, inversely to 4-nitrophenyl *N*, *N'*-diacetyl- β -D-chitobiosis (Table 2).

Fig. 3 Optimum temperature of the chitinase of *S. rhizophila* G22

Fig. 4 Optimum pH of the chitinase of *S. rhizophila* G22

The activity of the enzyme against other substrates, including colloidal chitin, native chitin, chitosan, chitin glycol, glycol chitosan, laminarin and carboxymethylcellulose, was analysed. The enzyme demonstrated highest activity with colloidal chitin and this result was accepted as 100%. Lower activity of the enzyme was observed with chitin glycol (approx. 25%), powdered chitin (20%) and chitosan (approx. 15%). With regard to other substrates studied, the chitinase showed trace or zero activity (Fig. 5).

Adsorption of chitinase to substrates

Binding of chitinase from *S. rhizophila* G22 to colloidal chitin and chitin powder was measured at 15 °C for 30 min. To calculate how much enzyme was bound during the reaction, the activity of free chitinase had to be determined. Adsorption of the enzyme to colloidal chitin at the level of 69% was observed already after 5 min of reaction and the binding level was 75% after 30 min. A much higher level of unbound

Table 2 Substrate preference and kinetic parameters of the chitinase *S. rhizophila* G22

Substrate	Activity (U)	K_m (mM)	V_{max} (μ M/h)
4-Nitrophenyl β -D- <i>N</i> , <i>N'</i> , <i>N''</i> -triacylchitotriose	61	1.87	89.7
4-Nitrophenyl <i>N</i> , <i>N'</i> -diacyl- β -D-chitobioside	12	2.2	18
4-Nitrophenyl <i>N</i> -acetyl- β -D-glucosaminide	0	nd	nd

Fig. 5 Substrate preference of the chitinase of *S. rhizophila* G22

chitinase was observed in the case of powdered chitin, indicating lower adsorption of the enzyme (Fig. 6).

The effect of metal ions and other substances on chitinase activity

Of the metal ions used in the experiment, only manganese at 1 and 5 mM and calcium at 5 mM were activators of the enzyme activity. The majority of the tested metal ions inhibited the enzyme. The biggest loss of activity—approx. 85%—was caused by Fe^{3+} and Cu^{2+} ions. Co^{2+} and Zn^{2+} ions caused a 20–40% inhibition. 5 mM Mg^{2+} and Ba^{2+} ions caused only a 10% inhibition of the enzyme (Table 3).

Fig. 6 Adsorption of the chitinase of *S. rhizophila* G22 to substrates

Triton X-100 was an extremely effective activator, leading to a 1.5 times increase in activity of the enzyme. The presence of Tween 20, urea and DMSO also stimulated the activity of the studied chitinase, by as much as 40%. SDS occurred to be a strong inhibitor of the activity of the enzyme and EDTA was demonstrated to be a partial inhibitor. A decrease in enzyme activity was also observed in the presence of iodoacetamide and PMSF (Table 4).

Sequence analysis of the chitinase-coding gene from *S. rhizophila* G22 bacteria

The gene encoding chitinase *S. rhizophila* G22 (sequence ID LC497040) is 2109 bp long. Comparison of sequences in Blast (<https://blast.ncbi.nlm.nih.gov>) alignment with chitinase genes derived from other *Stenotrophomonas* bacteria: *Stenotrophomonas* sp. LM091 (CP017483.), *S. rhizophila* strain DSM14405 (CP007597) and strain QL-P4 (CP016294) as well as *S. maltophilia* (AF014950) were obtained. Sequence coverage ranged from 99 to 100%, with sequence identity from 92.6 to 86.3% and with *E* value = 0.

The analysis of the amino acid sequence of the catalytic domain demonstrated that the analysed chitinase type A belongs to the family 18 of glycosyl hydrolases (GH18), type II. The structure of this domain is an eight-stranded alpha/beta barrel with a pronounced active-site cleft at the C-terminal end of the beta-barrel (<https://www.cazy.org/>). In the deduced amino acid sequence, a characteristic of hydrolases from this family of DxDxE motif occurs at position 465–469 aa, with aspartic acid important for catalysis. In

Table 3 The effect of metal ions on chitinase activity

Metal ions	Concentration (mM)	Relative activity (%)
Control	–	100
Ca ²⁺	1	100
	5	130
Mn ²⁺	1	123
	5	145
Mg ²⁺	1	101
	5	90
Ba ²⁺	1	91
	5	90
Zn ²⁺	1	81
	5	63
Co ²⁺	1	86
	5	68
Cu ²⁺	1	21
	5	14
Fe ²⁺	1	22
	5	16

Table 4 The effect of substances on chitinase activity

Substances	Concentration	Relative activity (%)
Control	–	100
PMSF	1 mM	89
	3 mM	53
Jodoacetamid	1 mM	67
	3 mM	51
Triton X-100	0.1%	122
	3.0%	149
Tween 20	0.1%	100
	3.0%	131
Urea	1 mM	117
	10 mM	131
DMSO	0.1%	119
	3.0%	137
SDS	1 mM	62
	10 mM	0
EDTA	5 mM	59
	10 mM	44

addition to the catalytic domain present in position 302–687 aa, the following sequence can be distinguished: chitin-binding domain—ChtBD3 (49–94), PKD (107–196) and the FN3 domain (203–280 aa) as presented in Fig. 7, according to: <https://smart.embl-heidelberg.de>.

Discussion

Stenotrophomonas bacteria are extremely active in the decomposition of non-soluble biopolymers, such as keratin and chitin which makes them highly useful in ecosystems (Jankiewicz et al. 2016). The study presented here was carried out on extracellular chitinase synthesised by the rhizosphere bacterium *S. rhizophila* G22. This is the first report providing the full characteristics of chitinase synthesised by this bacterial species.

A high chitinolytic activity was confirmed already after 3 days of culture at 28 °C. The chitinase was purified to homogeneity by chromatographic techniques and used for biochemical characterization. The molecular weight of chitinase from G22 bacteria measured with SDS-PAGE was approx. 50 kDa. It is by approx. 22 kDa lower than the value deduced on the basis of the gene sequence – 72 kDa. The results of this study and available literature data do not allow unequivocal definition of post-translational processing of the enzymatic protein. In this case, only the splitting-off of the 43 aa-long signal peptide is clear. Similar results are presented for chitinase from *Stenotrophomonas maltophilia* 34S1 and N4 (Kobayashi et al. 2002; Jankiewicz and Swiontek Brzezinska 2015). The molecular weight of *S. rhizophila* G22 chitinase precursor protein is similar to the molecular weight of chitinase synthesised by *Streptomyces olivaceoviridis* ATCC 11,238 (70 kDa)—a precursor protein for chitinases with molecular weights of 30 kDa and 20.5 kDa (Romaguera et al. 1992).

Zymograms obtained in this study demonstrate that *S. rhizophila* G22 synthesizes only one isoform of chitinase active under experimental conditions. Similar results were obtained for *S. maltophilia*. It is worth mentioning that there are two coding sequences found in genomes of those *Stenotrophomonas* genus bacteria in which conservative catalytic domains are located, including the approx. 2109 bp-long gene analysed in this study and the other one—1173 nucleotide-long, possessing no chitin-binding domain (Suma and Podile 2013). More isoforms of chitinases were described in several other bacterial species, mostly belonging to the genera *Bacillus* and *Streptomyces* (Alam et al. 1996; Rabeeth et al. 2011). The well-characterised chitinolytic machinery of *Serratia marcescens* was recognised as a model system for enzymatic degradation of insoluble chitin (Vaaje-Kolstad et al. 2013).

The optimum temperature for chitinolytic activity of the analysed enzyme was 37 °C. It is a lower value compared to literature data indicating an optimal temperature of 40 °C (Suma and Podile 2013), 45 °C (Jabeen et al. 2018) or 50 °C (Sosnowska et al. 2018) for chitinases from *S. maltophilia*. A similar result—an optimum in the range of 35–40 °C—was determined for *Streptomyces luridiscabiei*

Fig. 7 Organisation of domains in a chitinase molecule

U05 (Swiontek Brzezinska et al. 2019). There are also chitinases active at much higher temperatures, such as chitinase from *Paenibacillus timonensis* with optimum temperature of 80 °C (Yahiaoui et al. 2019).

The analysed enzyme demonstrated chitinolytic activity over a relatively broad range of pH. However, it had a higher activity in acidic environment, with optimum pH of 5.9. Similar results—optimal pH 6.0 and optimal temperature 40 °C—were obtained for recombinant BsChi-chitinase from *Bacillus subtilis* (Wang et al. 2018). Literature data suggest that the majority of bacterial chitinases reach their activity optimum at acidic or neutral pH (Senol et al. 2014), but there are also bacteria that synthesise alkaline chitinases, for example that of *Streptomyces* sp. CS495 with pH optimum of 12. The alkaline and thermostable chitinase synthesised by *Bacillus pumilus* JUBCH08 is an interesting example (Bhattacharya et al. 2016).

Chitinolytic enzymes are classified according to the position of the hydrolysed bond in a chitin molecule. The synergistic effect of endochitinase and exochitinase allows effective hydrolysis of resistant and non-soluble chitin (Vaaje-Kolstad et al. 2013; Zhu et al. 2016; Chu et al. 2019). Our results indicate that *S. rhizophila* G22 chitinase should be classified as an endochitinase, because its activity is higher in the case of substrates with a longer sugar chain. The kinetic test results obtained are also important. The obtained K_m value is much lower for 4-nitrophenyl β -D-*N,N,N'*-triacylchitotriose than for 4-nitrophenyl *N,N'*-diacyl- β -D-chitobioside, which indicates that the substrate binds more strongly to this enzyme. That result is consistent with literature data for several *S. maltophilia* bacterial strains (Suma and Podile 2013). On the other hand, exochitinase is synthesised by, among others, *Pseudoalteromonas* sp. DL-6 (Wang et al. 2016).

Chitinases isolated from various bacterial strains use different spectra of polysaccharide substrates or their derivatives. According to the obtained results, the enzyme did not show a broad substrate-specificity for polysaccharide derivatives. Substrates easily hydrolysed by the enzyme were: colloidal chitin and chitin glycol and—to much lesser extent—powdered chitin chitosan and laminarin. The analysed chitinase did not hydrolyse glycol chitosan, CMC or xylan. Similar to the enzyme analysed

by us, chitinase PbChi67 from *P. barengoltzii* hydrolysed colloidal chitin and chitin glycol. Chitosan and CMC were resistant substrates for chitinase PbChi67, similar to the enzyme analysed by Fu et al. (2016). On the other hand, chitinase *Streptomyces chilikensis* RC1830 demonstrated broad substrate-specificity, besides colloidal chitin effectively hydrolysing also xylan, CMC, starch and pectin (Ray et al. 2019).

The obtained results show that chitinase from *S. rhizophila* was adsorbed after 30 min on colloidal chitin much more efficiently (75%) than on powdered chitin (37%) obtained from shrimp shells and thus with a dominance of the α -chitin form. Suma and Podile (2013) observed StmChiA preference for α - and β -chitin at 42%. Similar results were also obtained for recombinant CHIT46 chitinase (Jun-Jin Deng et al. 2019). Detailed studies of chitin α and β adsorption conditions were carried out on *Serratia marcescens* chitinase (Vaaje-Kolstad et al. 2005).

Metal ions may be engaged in stabilisation of molecular structures of proteins and catalytic activity of numerous enzymes. Heavy metal ions often cause denaturation of enzymatic proteins. In the case of *S. rhizophila* G22, increased activity of chitinase was observed only in the presence of calcium and manganese ions. Calcium ions also activated chitinase obtained for *S. maltophilia* (Shaikh et al. 2018). Manganese ions activated chitinase from *Serratia marcescens* (Zarei et al. 2011). On the other hand, in the case of chitinases from *Streptomyces griseus* MTCC 9723 and *BsChi* from *B. subtilis* Mn ions caused an inhibition of its activity (Rabeeth et al. 2011; Wang et al. 2018).

Enzymes may be classified based on their susceptibility to various inhibitors. Inhibition of activity of the analysed chitinase in the presence of iodoacetamide and PMFS may indicate the presence of cysteine moieties in the enzyme molecule, playing an important role in its activity. This supports previous observations for *S. maltophilia* (Jankiewicz and Swiontek Brzezinska 2015). Other inhibitors, but also those specific for cysteine moieties (pCMB and NEM), inhibited the activity of chitinase *Paenibacillus timonensis* strain LK-DZ15 (Yahiaoui et al. 2019).

The ionic detergent (SDS) and non-ionic (Tween20 and Triton X-100) detergents also affected activity of the analysed chitinase.

Similar observations were made for chitinases from *Cohnella* sp. A01 and *Bacillus subtilis* (Aliabadi et al. 2016). On the other hand, chitinases from *Aeromonas schubertii* bacteria were resistant to SDS (Liu et al. 2009).

As mentioned above, there are two genes with distinguishable catalytic domains of chitinase in the *Stenotrophomonas rhizophila* genome. The gene composed of 1173 nucleotides has no chitin-binding domain, which prevents hydrolysis of insoluble chitin by the enzyme, the product of this gene. The analysis of nucleotide sequence of 2109 bp demonstrated the presence of domains typical for GH 18 family chitinases: catalytic Gly_18, chitin-binding—ChtBD3, type-III fibronectin—FN3 and polycystic kidney disease domain—PKD domain. Chitinase synthesized by *S. rhizophila* is a multimodular enzyme, similar to the previously described *S. maltophilia* chitinase with identical domain arrangement. A detailed analysis of the *Stenotrophomonas maltophilia* chitinase sequence was conducted by a group of researchers led by Kobayashi et al. (2002) and recently by Bhuvanachandra (2018). The latter authors conducted studies on molecular docking and described the interaction between the substrate (DP6) and StmChiA chitinase.

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Author contributions UJ, BB and MSB and MF designed the study. UJ, BB and MSB performed the research. UJ analysed the data and wrote the paper. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest. No grants were received.

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

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