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Biotechnology of Chitosanolytic Enzymes

– Review –

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CHITOSAN, CHITOOLOGOSACHARIDES, AND THEIR INDUSTRIAL APPLICATIONS

Chitosan and its oligomer have been of interest in the past few decades due to their broad range of medical, agricultural and industrial applications. This potential is associated with their characteristics being reported as antibacterial, antifungal, hypocholesterolemic and antihypertensive actions. Chitosan has been used as feed additive since 1983, and as food quality enhancer in several countries (1-4).

Chitosan, a deacetylated form of chitin, is a polymer of heteropolysaccharide which is composed of N-acetyl glucosamine and N glucosamine β (1-4) 2 acetamido-D-glucose and β (1-4) 2 amido-D-glucose units, with the latter being usually 80% or more. Generated from abundantly renewable sources of chitin, chitosan and its oligomers, are soluble in acid solutions and water. The three types of reactive groups, i.e. one amino group at the C-2 and hydroxyl groups at C-3 and C-6, respectively are important for the wide variety of it's applications to preserve foods from microbial deterioration, formation of biodegradable films, recovery of waste material from food processing discards, purification of water and clarification and deacidification of fruit juice (1,2,5).

The chitooligosaccharides depolymerized from chitosan are demanded because of their high solubility in which the application is even widened and absorption *in vivo* is improved. Chitosan oligomers, highly valuable forms of chitosan, can be made by either chemical or enzymatic hydrolysis of chitosan. Acidic hydrolysis, which is partial hydrolysis of chitosan using concentrated HCl, produces small amount of oligosaccharides and large amount of monomeric D-glucosamine. This means that the process results in a high amount of oligomers with a low degree of chitosan polymers, which vary from monomer to trimer. The production of high degree products, on the other hand, is low (5,6). High degree of polymerization (DP) products are more desirable than the monomer forms due to their active biological properties. Efficient production of high DP oligomer products can only be possible with enzymatic hydrolysis of chitosan (2,7). In particular, enzymatic hydrolysis of chitosan is very useful for producing di-, tri-, and tetra-chitooligosaccharides mainly. In addition to its efficiency, enzyme hydrolysis also results in high quality and well defined products because the process is relatively controlled and non degrading (6). Certain enzymes such as chitinase and lysozyme have been reported to be able to hydrolyze chitosan. Several other enzymes such as protease, glucanase, lipase, tannase and papain, also have this property. However, chitosanase is the most efficient to hydrolyze chitosan and produce chitosan oligomer (3, 4,7).

ENZYMES CAPABLE OF HYDROLYZING CHITOSAN

Chitosanases (EC 3.2.1.132 or EC 3.2.1.99) are enzymes that catalyze endohydrolysis of β-1,4-linkages between N-acetyl-D-glucosamine and D-glucosamine residue in a partly acetylated chitosan. They are grouped into the glycoside hydrolase family. According to the Enzyme Commission, chitosanase acts only on polymer with 30~60% acetylation. Chitosanases which are mostly extracellularly produced have specific hydrolytic action pattern depending on the microorganism and the degree of acetylation of the substrate. The enzyme differs from chitinase; i.e. attacking lower N-acetylated polymer and having lower apparent molecular mass (8-10).

Based on their cleavage specificity, chitosanases can be classified into three classes, i.e. class I, which can split GlcNAc-GlcN in addition to GlcN-GlcN; class II which can split GlcN-GlcN; and class III which can split GlcN-GlcNAc in addition to GlcN-GlcN (9,11). Based on sequenced chitosanase genes, they may belong to either one of three glycoside hydrolase families, i.e. family 46, 75 and 80. The most studied chitosanases such as *Streptomyces* sp. strain N174, *Nocardioides* sp. strain N106, *Bacillus* are members of family 46 of glycosyl hydrolases (10-12). Chitosanases from the plant pathogenic fungus such as *Fusarium solani*, are totally different from those from bacteria. They are not classified as family 46 gly-

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cosyl hydrolase but as glycoside hydrolase family 75 (13, 14). Other members of the family include chitosanase from *Aspergillus fumigatus*, entomopathogenic *Metarhizium anisopliae*. Glycoside hydrolase 80 share sequence homology with the chitosanase from *Matsuebacter chitosanotabidus* and *Sphingobacterium multivorum* (14,15)

Among chitosanases that have been studied so far, those of glycosyl hydrolase family 46 are the only chitosanases whose three-dimensional structure has been determined. The enzyme, that is classified as an inverting enzyme, shows the mechanism of the hydrolytic reaction involving two carboxylic acid residues, which directly participate in the catalytic reaction. One of the carboxylic acid residue acts as a proton donor with its unionized form, while the other activates water molecule which then attack the C-1 carbon of the sugar residue located at the catalytic site. Glu-22 and Asp-40 are the catalytic residues (11) while tryptophan residues are most likely to play important roles in the protein stabilization (16).

Chitosanases are produced by many organisms such as actinomycetes (2), fungi (11,15,17) plants (2,8) and bacteria (2,12,18). Among bacteria, *Bacillus* and *Streptomyces* chitosanases are the most frequently studied (9,11,12). Bacterial chitosanases have received special attention, because they are important for the maintenance of the ecological balance and have been used to determine the mechanism of chitosan hydrolysis at biochemical and molecular levels. Compared to the numerous reports on the primary structure and functions of chitinase, information on chitosanases is still relatively limited.

Most of bacterial chitosanases are reported to be induced by chitosan in the media and play a role in the degradation and utilization of exogenous chitosan. Chitosanases can be included in the important complex enzymes related to recycling processes of chitin in nature. The enzymes provide the microorganism producers a competitive advantage over fungi which contain chitosan in their cell walls. Several fungi excrete chitosanase when grown in media containing readily metabolized carbon sources such as glucose, whereas addition of chitosan to the media resulted in severe inhibition of the cell growth (19). The existence of considerable amount of chitosan in their cell wall, implies the physiological role of fungus chitosanase in the cell wall associated chitosan metabolism. The enzyme might play an important role in cellular activities such as cell division and autolysis. The plants which excrete chitosanase enzymes use this in plant defense mechanism against pathogenic fungi (8).

Chitosanases that catalyze the endo-type cleavage of chitosan polymers are potentially useful in the large scale production of chitosan oligomers which have beneficial biological activities. The properties of chitosanase produced from several microorganisms are described as follows (Table 1 and Table 2).

Chitosanase excreted by the fungus *Aspergillus* sp Y2K is a molecule of 25 kD, with optimum pH of 6.5 and optimum temperature being 65~70°C (17). By endosplitting activity, the chitosanase hydrolyze chitosan to produce chitooligomers, with chito-triose, chito-tetraose and chito-pentaose as the major products. A novel gram negative bacteria *Matsuebacter chitosanotabidus* 3001 excreted a 34 kD chitosanase which works well at acid condition with optimum pH being 4 and optimum temperature being 30~40°C (15). The N terminal amino acid sequence was considered unique, since it did not show any significant homology to the known chitosanase. A novel chitosanase producing bacterium belonging to the genus *Pseudomonas* was reported by Yoshihara et al. (23). The 35 kD enzyme was active at acid condition, with optimum pH 4. The enzyme split chitosan in endo-manner, and the shortest oligomer to be attacked is the tetramer. Some of chitosanase hydrolyze not only chitosan, but also other

Table 1. Properties of purified chitosanase from different sources

Microorganisms	MW (kD)	Opt pH (Stability)	Opt temperature (Stability)	Inhibitor	References
Bacillus cereus S1	45	6 (6~11)	60°C (<60°C)		10
Bacillus PI-7S	43(SDS-PAGE)[1]; 25 (GF)[1]	6.8 (3.3~7.4)	45°C (<45°C)	Hg^{2+}, Ag^{+}, p-CMB	9
Bacillus sp. strain CK-4	29	6.5	60°C	Cu^{2+}	20-22
Pseudomonas sp. H-14	35	4 (4~7)	50°C (20~50°C)		23
Aspergillus sp. Y2K	25 (SDS-PAGE)[1] 22 (GF)[1]	6.5 (4~7.5)	65~70°C (55°C)	Hg^{2+}, Cd^{2+}	17
Amycolatopsis sp. CsO-2	27	5.3 (4.5~6)	55°C (0~50°C)		24
Acinetobacter sp. strain CHB 101	37 & 30	5~9	40°C		25
Enterobacter sp. G-1	50		50°C (20~50°C)		26
Fusarium solani f.sp. phaseoli	36	5.6			19
Matsuebacter chitosanotabidus 3001	34	4.0	30~40°C	Ag^{2+}	15

[1]MW was determined through SDS-PAGE or GF (Gel Filtration).

ubstances such as Carboxymethyl Cellulose (Table 2). The gram negative *Myxobacter* sp. AL-1 produced a 32~35 kD molecule with dual chitosanase-cellulase activities 27). The chitosanase part was more active at 70°C than at 42°C, while the cellulase activity was more active at 42°C than at 70°C. This implies that this enzyme posseses different catalytic sites for attacking the two related but different polysaccharide substrates (19).

MECHANISM OF REACTION

It has been reported that the N acetylglucosamine residues in chitosan may not be important in the recognition of the substrate by the enzyme. The activity of some enzymes from *Bacillus* sp. is usually increased as the degree of deacetylation of chitosan is increased. Chitosanase mainly hydrolyzes chitosan into glucosamine oligomers above $(GlcN)_3$ in a random splitting manner in the initial stage, and it hydrolyzes the oligomers above $(GlcN)_6$ which are produced by the previous /primarily hydrolysis into $(GlcN)_2 \rightarrow (GlcN)_4$ rapidly in the final cleavage (9,10,26).

Due to their cleavage properties, chitosanases are considered to relate structurally and functionally to lysozyme whose substrate is an alternating polymer of N-acetylglucosamine and N-acetylmuramic acid. In fact, X ray crystal structure of *Streptomyces* sp. N174 chitosanase with its molecular weight of 25811 Da was reported to be similar to that of hen egg white lysozyme in their catalytic core (11,16). In hen egg white lysozyme, several tryptophan residues are localized to the substrate binding cleft and play important roles in lysozyme reaction. The importance of tryptophan residues in substrate binding had been recognized also for other carbohydrolase because of their ability of stacking and non polar interaction with the pyranose

Table 2. Substrate specificity of the chitosanase

Chitosanase source	Substrate specificity		Reference
	Macromolecule	Oligochitooligisaccaride → end products	
Bacillus cereus S1	Soluble chitosan (100%) CMC (20%) Colloidal chitosan (30%)	100% DA chitosan → chitobiose (27.2%) + chitotriose (40.6%) + chitotetraose (32.2%) Chitotetraose → chitobiose + chitotriose	10
Bacillus PI-7S	99% DA chitosan	Chitotetraose → chitotriose + GlcN Chititriose → chitobiose + GlcN	9
Bacillus sp. strain CK4 (thermostable)	99% DA soluble chitosan (100%) 88% DA soluble chitosan (88%) 71% DA soluble chitosan (71%) 53% DA soluble chitosan (53%) 99% DA coll. chitosan (20%) 88% DA coll. chitosan (34%) 71% DA coll. chitosan (67%) 53% DA coll. chitosan (87%) Glycol chitosan (20%)	Chitosan → $(GlcN)_4$	21,22
Pseudomonas sp. H-14	100% DA soluble chitosan (100%) 87% DA soluble chitosan (84%) 75% DA soluble chitosan (54%) 62% DA soluble chitosan (36%) Glycol chitosan (4%)	Tetramer/heptamer → dimer + trimer	23
Burkholderia gladioli strain CHB 101	Low acetyl chitosan (0~30%)	Pentamer GlcN	13
Amycolatopsis sp. Cs)-2	70~100% DA chitosan	Pentamer → dimer + trimer	24
Fusarium solani f. sp	Glycol chitosan (100%) 70% DA chitosan (93%) 100% DA chitosan (57%) Glycol chitin (9,4%) Carboxymethyl cellulose (2,5%)		28
Aspergillus sp. Y2K	O-carboxymethyl chitin	Chitosan (>chitohexaose) → chitotriose, chitotetraose, chitopentaose	17
Mycobacter sp. AL-1 (chitosanase-cellulose)		>6 unit of GlcN	27
Enterobacter sp. G-1	Glycol chitin Colloidal chitin 80% colloidal chitosan	$(GlcNAc)_4 \rightarrow 2\ (GlcNAc)_2$ or $(GlcNAc)_3 + GlcNAc$ $(GlcNAc)_5 \rightarrow (GlcNAc)_3 + (GlcNAc)_2$ $(GlcNAc)_6 \rightarrow (GlcNAc)_4 + (GlcNAc)_2$ or $2(GlcNAc)_3$	26

ring of the substrate. The mutation studies of chitosanase of *Streptomyces* sp. N174, show the importance of tryptophan residue in making various interactions with hydrophobic and carboxyl side chain at the hydrophobic core. The mutation of tryptophan residue resulted in partial collapse of the side chain interaction (16).

MICROORGANISMS FROM HIGH TEMPERATURE ENVIRONMENT AND THERMOSTABLE CHITOSANASE

The interest on microorganisms isolated from high temperature environment is increasing because of its application for biotechnology and industries. These microorganisms produce thermostable enzymes and other proteins which can function optimally at high temperatures and perform high stability at elevated temperatures. Thermophilic enzymes also posses great resistance to proteolysis, detergents and chaotropic agents (29,30).

Microorganisms that can live at high temperature or heat-loving microorganisms are called thermophiles. Some researchers define thermophilic microorganisms more specifically. Thermophile microorganisms are defined as those lived at 45~65°C, and have optimum growth temperature of >50°C. On the other hand, extreme thermophiles are those capable of growing at 65~85°C with optimum temperature of >65°C. Microorganisms capable of living above temperature of 85~110°C, and have optimum growth temperature of above 100°C are classified as hyperthermophiles (18,29). Biotopes of thermophiles are usually found naturally within volcanic area and other geothermal locations. However, this organisms can also be isolated at other areas such as garden soils and glaciers.

Thermostable chitosanase are active at 60~100°C. Report on the gene encoding this enzyme has been documented (20). The chitosanase from *Bacillus* sp. Strain CK4 degraded soluble, colloidal and glycol chitosan, but not chitin and β linked polymers. Chitosan was degraded to $(GlcN)_4 \rightarrow (GlcN)_2$ as the major product. The optimum temperature and pH were 60°C and pH 6.5. Ca and Mn have been reported to increase the enzyme activity (21,22). Yoon et al. (20) reported analysis of gene encoding thermostable chtosanase from *Bacillus* sp. strain CK4. The gene is composed of a 822 bp open reading frame which encodes a protein of 242 amino acids. The recombinant chitosanase shows an optimum temperature of 55°C but, is highly thermostable, with a half life of 90 minutes when incubated at 80°C. Yoon et al. (21) reported study on site directed mutagenesis to identify amino acid residues responsible for thermostability of chitosanase from *Bacillus* sp. CK4. Mutation of Asp-66 caused a drastic loss of the chitosanase catalytic activity indicating that Asp 66 is essential for the enzyme reaction. The thermostable chitosanase contain three cysteine residues at positions 49, 72 and 211. When the cysteine residue at 211 position was substituted by serine or tyrosine, the mutant enzyme was less stable towards heat, protein denaturants and organic solvents, while similar substitution of other cystein residues did not change the thermal stability. The cystein at 211 position appeared to play an important role in the conformation stability of the chitosanase (21).

We have screened a number of thermophilles producing chitosanase from geothermal areas in Indonesia. The extra cellular enzymes from a *Bacillus licheniformis* MB-2 showed optimum temperature at 80°C and is thermostable (Fig. 1 and 2). When heated at 90°C, the enzyme still

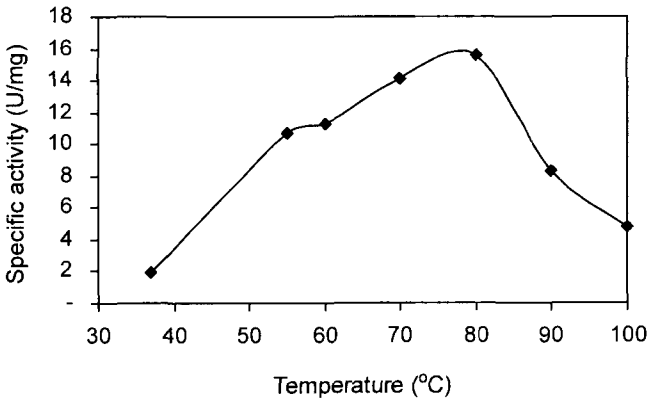

Fig. 1. Optimum temperature of chitosanase MB-2.
The *Bacillus licheniformis* MB-2 bacteria was grown in medium consisted of 0.4% colloidal chitosan, 0.5% $MgSO_4$, 0.3% KH_2PO_4, 0.7% K_2HPO_4, 0.25% yeast extract and 0.25% casiton, pH 7.0. Enzymes was precipitated using 80% saturated NH_2SO_4, followed by dialysis (membrane with molecular weight cut-off of 10,000). Enzyme assay follows that of Uchida & Ohtakara (12). 1 unit of enzyme was defined as the amount of the enzyme which produces 1 μmol of reducing sugar as glucosamine per minute.

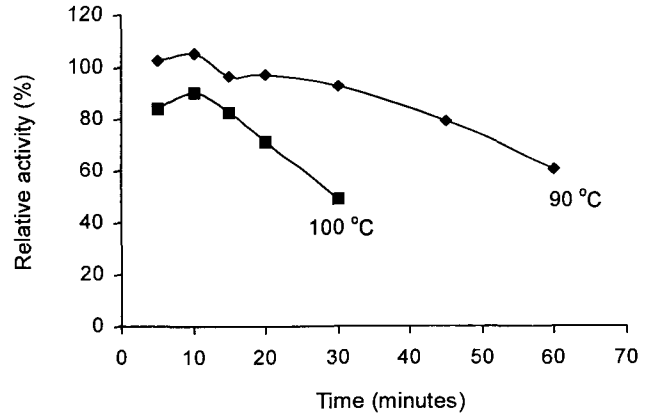

Fig. 2. Heat stability of chitosanase MB-2.
The enzyme was incubated at the temperature of 90°C and 100°C at various time in the absence of substrate, and the residual activities were measured under standard assay condition using 1% soluble chitosan, pH 6.0.

retained 50% of activity after 1 hour, while the half life at 100°C was 25 minutes.

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