PURIFICATION OF CHITINASE PRODUCED BY *Streptomyces* BRI 36

SELYVESTER @ JULKARNAIN KUNDIAN

THIS DISERTATION IS BEEN PRESENTED TO FULLFILL PART OF THE REQUIREMENT TO OBTAIN BACHELOR OF SCIENCE WITH HONOURS

BIOTECHNOLOGY PROGRAMME
SCHOOL OF SCIENCE AND TECHNOLOGY
UNIVERSITY MALAYSIA SABAH

OCTOBER 2006

PUMS 99:1

UNIVERSITI MALAYSIA SABAH

BORANG PENGESAHAN STATUS TESIS@

JUDUL: PURIFICATION OF CHITINASE PRODUCED BY STREPTOMYCES BRI 36

Ijazah: SARJANA MUDA SAINS (BIOTEKNOLOGI)

SESI PENGAJIAN: 2003 – 2006

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ACKNOWLEDGEMENT

First of all I am very grateful to the almighty God, with His blessing and protection has enabled me to be where I am now.

I consider it as my personal scientific achievement when I finished this final year project, so I am very grateful and thankful to Lord in heaven for all the blessings and kindness being given to me all the time.

A million thanks to my parents and my dear family for their support and encouragement given to me during my study in UMS, all the support and sacrifices made for me is deeply appreciated. Special thanks to Elu and Dellselwyn, thank you for being patient, understanding and supportive to me.

I am very thankful for my supervisor Yg. Berbahagia Professor Datuk Dr. Kamaruzaman Ampon for accepting me as his student in doing this final year project and also for the advises along the project period was conducted.

I would also like to thank you the School of Science And Technology's Dean for all his support especially for giving me the permission to work in the laboratory beyond office hour. Thank you also to all Biotechnology lecturers for the support and valuables lesson being thought through out the years in UMS.

Thank you to Mr. Chong Tong Seng and Mr. Lum Mok Sum for their valuable guidance and lessons being thought to me in conducting this project. Thank you Mr. Adrian Ng from the Biotechnology research Institute for supplying the bacteria needed for this project and thanks also for the chemicals that being given to me. Thanks also to all other Master student that has shared their knowledge and know-how with me. To the entire lab assistant especially Mr. Musbah, thank you for your support and corporation and making my life in lab fun and easy.

To all my friends, Kipin, Wald, Joe, Mus, Hendricks, Khairi, Jason, Bryan, Liza, Rudz, all the Biotech students class of 2003 and my junior, I really enjoyed your company and I really appreciate those wonderful and fun times we've spent together during my study in UMS. Thank you all and may God Bless you all.

ABSTRACT

Spore suspension of *Streptomyces* BRI 36 was inoculated in 100 ml of enzyme production media in two batches. The synthesis of an extracellular Chitinase by *Streptomyces* BRI 36 was induced by using colloidal chitin as its sole carbon source. Chitinase was isolated from the culture filtrate and purified by acetone precipitation, Sephadex G-25, Sephadex G-50 and Sephadex G-100 gel filtration. Enzyme assay was done by using colloidal chitin stained with Remazol Brilliant Blue® as substrate. Final yield was about 21% for batch 1 and 9% for batch 2. Total protein was estimated by using modified Lowry method and given estimation of 5.15 mg for batch 1 and 1.88 mg for batch 2.

ABSTRAK

Ampaian spora *Streptomyces* BRI 36 diinokulasikan ke dalam 100 ml media penghasilan enzim, ianya dilakukan dalam 2 set. Penghasilan enzim Chitinase oleh *Streptomyces* BRI 36 dirangsang dengan menggunakan ampaian chitin sebagai sumber karbon utamanya. Proses pengasingan dan penulenan enzim Chitinase dijalankan dengan menggunakan kaedah pemendakan dengan aseton dan pemisahan menggunakan gel pemisah Sephadex G-25, Sephadex G-50 dan Sephadex G-100. Proses analisis aktiviti enzim dijalankan dengan menggunakan ampaian koloidal chitin yang diwarnakan dengan Remazol Brilliant Blue®, sebagai substrat. Enzim Chitinase yang diperolehi merupakan enzim yang mempunyai saiz molekul yang besar. Perolehan akhir bagi set 1 adalah sebanyak 21% dan set 2 pula sebanyak 9%. Jumlah protein akhir yang diperolehi adalah dianggarkan sebanyak 5.15 mg bagi set 1 dan 1.88 mg bagi set 2. Jumlah protein akhir ditentukan dengan menggunakan kaedah Lowry yang telah diubahsuai.

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LIST OF ABBREVIATION

Na_2HPO_4 – di sodium hydrogen orthophosphate anhydrous

K_2HPO_4 – di potassium hydrogen phosphate (potassium phosphate dibasic)

KH_2PO_4 – potassium dihydrogen phosphate (potassium phosphate monobasic)

NaCl – Sodium Chloride

$Cacl_2$ – Calcium chloride

NH_4Cl – Ammonium chloride

$MgSO_4$ – Magnesium Sulphate

GlcNac - N-acetylglucosamine

CC-RBB – colloidal chitin-Remazol Brilliant Blue®

RBB – Remazol Brilliant Blue®

mg – milligram

µg – microgram

ml – milliliter

µl – microliter

g- gram

U – units

Abs – Absorbance

°C – Degree Celsius

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CHAPTER 1

INTRODUCTION

Malaysia, especially Sabah is well known as one of the 12-mega biodiversity areas in the world hence there is always a possibility of finding a new species of organisms or microorganisms. The Biotechnology Research Institute of University Malaysia Sabah previously isolated *streptomyces* BRI 36. It has not been confirmed as a new species of *Streptomyces* but this bacterium show high similarities with actinobacteria based on the 16S-rDNA sequences. *Streptomyces* BRI 36 was isolated from the soil taken at Merajah River in the district of Kota Belud, Sabah. This bacterium shown high production of chitinase, based on the visual inspection of the halo produced by their colonies on the chitin agar medium (CHDA).

Over 500 species of *Streptomyces* have been recognized and chitinase has been purified from various species of it. Each of the chitinase purified from the different species of *Streptomyces* has its own characteristics in term of molecular weight, optimum pH and specific activity.

The productivity of chitinase also differs among the species. *Streptomyces* is considered as the major producers of chitinase in soils, since it is a primarily soil organisms. *Streptomyces* also regarded efficient in the breakdown of chitin.

Chitinase is defined as an enzyme that catalyzes hydrolysis of N-acetyl-D-glucosaminide (1-4)-β-linkage in chitin and chitodextrins. Chitinase are synthesized by a vast array of organisms and being widely distributed in nature and plays an important role in the degradation of chitin. Microorganisms produce chitinases to digest chitin primarily to utilize it as a carbon and energy source. The possibilities of using Chitinase for biological control and for the exploitation of natural chitinous material has caught the attention of scientist in the last decades and many research are still been done on Chitinase and chitin derivatives.

Chitin is an insoluble linear β-1, 4-linked polymer of N-acetylglucosamine (GlcNac). Chitin is produced in enormous amount in nature second after cellulose. It is nearly always cross-linked to other structural components such as β-glucans in fungi and specific proteins in invertebrates. Chitin also constitutes the exoskeleton of insects, flying apparatus of insects, and tendons of crustaceans.

This research aim is to obtain a purified chitinase produced by *Streptomyces* BRI 36 with minimal loss of biological activity. The research objectives are to culture the *Streptomyces* for enzyme production, to devise a suitable method for purification and to devise a suitable enzyme assay for chitinase. The research scope is to do the purification using precipitation and column chromatography.

CHAPTER 2

LITERATURE REVIEW

2.1 Chitin

Chitin, (1-4)-linked 2-acetamido-2-deoxy-β-D-glucan is a natural, non-toxic, non-allergenic, antimicrobial and biodegradable. Chitin is an insoluble polysaccharide and it is the most abundant nitrogen-bearing organic compound found in nature other than carbohydrate. Many exoskeleton of organism compose of chitin. It is widely distributed among invertebrates. Chitin can be found in hydrozoa, the eggshells of nematodes, mollusk, arthropods, cuttlefish bone, and squid pen.

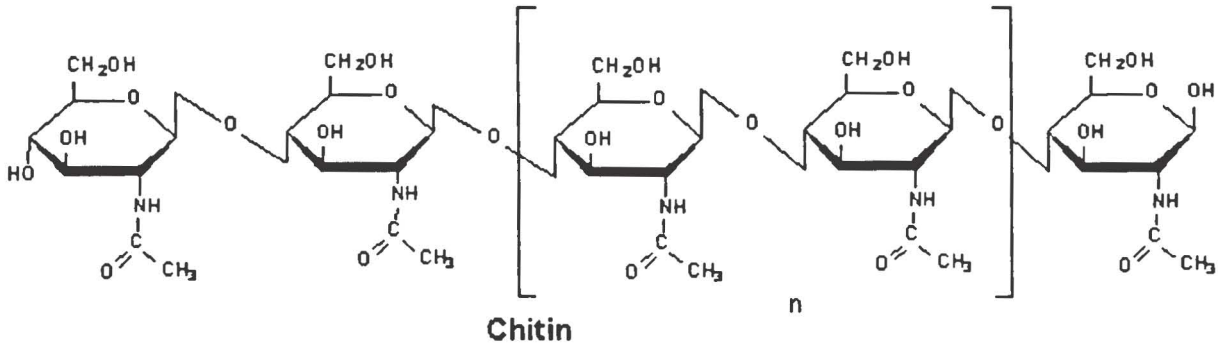

Figure 2.2 Chemical structure of chitin, a β-(1-4)-N-acetyl-D-glucosamine polymer.

Chitin can be processed into many derivatives such as chitosan. All of the derivatives have a potential application for biomedical materials. Wound dressing materials has been produced from chitin. N-acetyl-D-glucosamine (GlcNac), one of the end products of chitinase reaction with chitin, used for treatment of ulcerative colitis and other gastrointestinal inflammation disorder (Pichyangkura *et al*, 2002).

There are two classes of chitin based on the arrangement of chitin molecules; α-chitin and β-chitin. The α-chitin has an antiparallel molecule arrangement. The α-chitin has a poor solubility and reactivity with rigid crystalline structure because of the antiparallel arrangement of its molecules. The α-chitin is usually found in the calyces of hydrozoa, eggshells of rotifers, the radulae of mollusk and the cuticles of arthropods.

The β-chitin has a parallel arrangement of chitin molecules, where all their reducing ends pointed towards the same direction in a given microfibril. The β-chitin has a loose packing of sugar chains due to the parallel arrangement of the chitin molecules. The shells of brachiopods, cuttlefish bone, and squid pen and pogonophora tubes are composed of the β-chitin.

Many organisms have utilized chitin as a structural component of protective cell walls or exoskeletons. They are light and resistant composites with specific structural and mechanical properties. Chitin, in the form of microfibrils provides the high strength, which allows them to resists tensions and modulus. Chitin can attain different properties when it combined with other components of the cell walls or exoskeletons. A strong, pliable and flexible structure is obtained when proteins form covalent links with chitin. In insects, these properties allows movement and limit expansion. Chitin also forms conjugates with carotenoids thus gives color to tissues in insects (Herrera *et al*, 1999).

Chitin is not the most abundant component in the cell walls and exoskeletons, but the degradation of chitin can lead to the destruction of the whole structures. This is because chitin serves as the anchor around which the protective structures are made.

Synthesis of this protective structure involves the following steps: First the chitin molecules are synthesized intracellularly then the chitin molecules are being transported to the extracellular space. In its noncrystalline stage, parts of the noncrystalline chitin undertake chemical modification and associates with other molecules.

The unmodified chitin crystallizes and is covered by the rest of the components. The resulting structure matures into a composite, which provides protection to the organism.

Chitin biosynthesis is a complex process consisting of a series of enzymatic steps where the glucose unit is converted to N-acetylglucosamine, linked with uridine triphosphate and then transported within the cell in combination with dolichol phosphate, polymerized into chitin and covalently linked to proteins to form chitin microfibrils in the cuticle.

Chitin is synthesized differently in fungi and arthropods. In fungi the chitin synthase enzyme occurs as an inactive zymogen in vesicles called chitosomes and requires proteolytic activation while in arthropods this enzyme is membrane bound and catalyzes the additions of GlcNac units to a dolichol carrier. In insects, the chitin is then sclerotized and in crustaceans it is mineralized with calcium carbonate deposition in the cuticular canals (Palli *et al*, 1999).

2.2 Chitinase

Chitin can be completely hydrolyzed to free N-acetylglucosamine by chitinolytic enzymes. These enzymes commonly known as chitinases. Chitinases can be classified into two major categories; exochitinases and endochitinases according to where hydrolytic cuts happen in the chitin chains (Kupiec *et al*, 1998).

Endochitinase cuts chitin into oligosaccharides of differing chain lengths. The cuts occur randomly at internal sites, generating soluble low molecular mass multimers of GlcNac such as chitotetraose, chitobiose and the dimmer di-acetylchitobiose.

Exochitinase degrades oligosaccharides into di-acetylchitobiose and chitobiose. Exochitinase can be further divided into two sub categories, chitobiosidases and 1-4-β-N-acetylglucosaminidases. Chitobiosidases catalyzed the progressive release of Di-acetylchitobiose and the later cleave the oligomeric products of endochitinases and chitobiosidases into GlcNac monomers (Kupiec *et al*, 1998).

Chitinases are found in many organisms and their properties are closely related to their biological function. Based on the amino acid sequence similarity of chitinases from various organisms, five class of chitinase has been proposed. They are class I, class II, class III, class IV and class V. This class is grouped into two families known as family 18 and family 19.

Family 18 consists of class III and V chitinases. Class III is mainly plant and fungal in origin and class V is mainly comprised of bacterial chitinases. Family 19 is consists of class I, class II and class IV chitinases and all of them are of plant origin, except for chitinase that has been purified from *Streptomyces griseus* HUT 6037. Family 18 and family 19 chitinases are structurally unrelated to each other (Ohno T. *et al*, 1996).

Family 18 is the largest chitinase family, with about 180 members found in eukaryotes, prokaryotes and viruses. This family also contains enzymes such as chitodextrinase, an exochitinase, and endo-N-acetylglucosaminidases. Proteins of unknown function such as mammalian oviduct glycoproteins are also found in this family. Family 18 chitinase is an α / β barrel structure with eight parallel strands of sheet and eight return helices. The eight strands of the sheet bend into barrel structure with helices forming a ring toward the outside. Families 18 contain several runs of conserved amino acids found in the active site and include a Glu residue, which is crucial to the catalytic mechanism. The amino (N) terminal 147 residues form a distinct chitin-anchoring domain, which is found in many, but not all, family 18 chitinases. The α / β barrel domain is form by the residues 148 to 561 (Robertus *et al*, 1999).

Family 18 chitinases hydrolyze chitin with retention of the anomeric configuration at the cleavage point, implying a double displacement mechanism. Family 18 chitinases have a carboxylic residue which protonates the glycosidic oxygen in the first step of catalysis. Most of the family 18 chitinases have a modular structure with one or several

non-catalytic domains and can be found at their N- or C-termini. A number of these non-catalytic domains function as chitin binding domains.

Family 19 has more than 130 members, which are all from plant origin, except for the chitinases obtained from *Streptomyces griseus*. Chitinases of family 19 operates with the inversion of the anomeric configuration at the site of cleavage. Much family 19 chitinases display a modular structure within a N-terminal chitin binding domain and several members are made of a single catalytic domain with no ancillary domain. A good representation of family 19 chitinase is the chitinase obtained from barley. The structure of barley chitinase has been fully elucidated (Henrissat, 1999).

There are vast arrays of chitinase producer and each of the chitinases produced has its own physiochemical properties. The molecular weight of chitinases from plant and algae is estimated at about 30 kDa. Chitinases produced by mollusks, arthropods, fishes, amphibians and mammals has a molecular weight ranging from 40 kDa to 120 kDa. Bacteria and fungi produced chitinases with molecular sizes from 30 kDa to 120 kDa. Some chitinases are glycoproteins, such as the chitinases obtained from carrots, tobacco hornworm and silkworm and even from microorganisms like *Aeromonas* and *Rhizopus* (Koga *et al*, 1999).

All of the organisms can produce acidic and basic chitinases due to the wide range of pI values being observed. In higher plants the chitinases has a pI value of 3.0 to 10.0; pI values of chitinases from insects, crustaceans, mollusks and fishes are from 4.7 to 9.3

and chitinases in microorganisms has a value ranging from 3.5 to 8.8. The optimum pH for the chitinases from plants, animals and microorganism are at 3.5 to pH 9.0 and depends on the substrate used. Regarding thermal stability, some chitinase show a resistance to high temperature, 75°C to 80°C. This has been observed in chitinase purified from *Streptomyces thermoviolaceus* (Tsujibo *et al*, 1993) and *Bacillus* strain, MH-1 (Sakai *et al*, 1998).

Allosamidin is the substance known to inhibit the reaction of chitinases. It inhibits chitinase competitively. This inhibitor has similar structure with the intermediate of the substrate such as an oxazoline ring. Some metal ions can also inhibit chitinase like Hg^{2+} and Ag^{+} while Cu^{2+} can either inhibit or enhance chitinase.

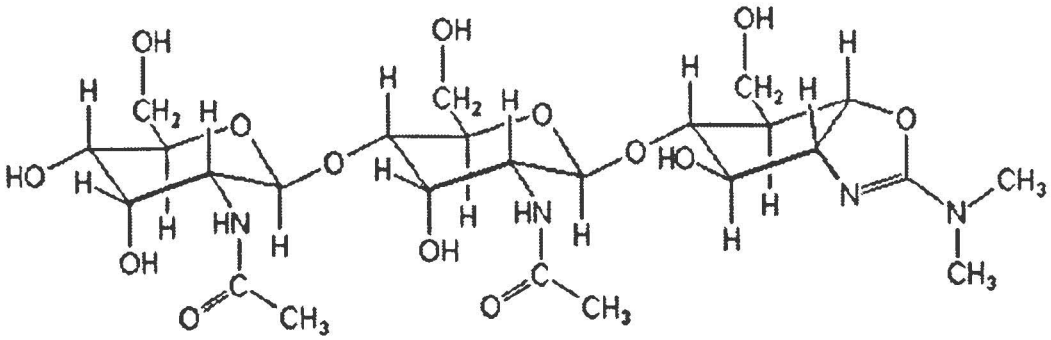

Figure 2.3 Structure of Allosamidin.

Previously chitinases have been purified from various species of *Streptomyces* and each of the chitinase obtained demonstrates different physicochemical properties. Some properties of the chitinases are summarized in the table given.

Table 2.1 Properties of chitinase from various species of *Streptomyces*

Species	Isozymes	Molecular Weight (kDa)	Isoelectric Point	Optimum pH	Optimum Temperature (°C)	Specific Activity
olivaceovidus	Endochitinase	46	-	-	-	0.34 U/mg
	Exochitinase	70	-	-	-	-
		92	-	-	-	-
Griseus HUT 6037	C1	27	7.7	4.5-6.0	55	131 U/mg
	C2	27	7.3	4.5-6.0	55	130 U/mg
	C	49	7.3	4.5-6.0	40	41 U/mg
kursanovii	Chi42	42	-	-	-	10 U/mg
Thermovidaceus OPC-520		40	3.8	-	-	82.5 U/mg
erythreus		30	3.7	5.0		-
Lydicus WYEC 108		32	-	-	-	-
		37	-	-	-	-

The reason for the present of isozymes in the bacteria is probably because of the diverse form of chitin found in nature.

Chitinases act by hydrolytically cleaving the β-glycosidic linkages between the GlcNac residues. Generally, the cut can occur in two ways, either with the retention of anomeric configuration in the product or with inversion. This depends on which family the chitinases belong. Family 18 chitinases will produced the N-acetylchito-

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