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Purification and Chitinolytic Characteristics of Chitinases A and B from *Serratiamarcescens*

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Abstract

In Serratiamarcescensthe enzymatic chitinolytic machinery of is one of the best that can convert insoluble polysaccharides.
This process includes four chitin-active enzymes: ChiA, ChiBand ChiC, an endo-acting non-processivechitinase, , two processivechitinases moving along chitin chains in opposite directions. Serratiamarcescensas a Gram-negative soil bacterium produces these three chitinases, ChiA, ChiB and ChiC which together enable Serratiamarcescens to perfectly degrade the insoluble chitin polymer. The data collected for the pseudotrisaccharideallosamidinshows a comparative inhibition including the cyclic pentapeptideargadin. ChiA, ChiBand ChiC play different roles during chitin degradation was confirmed by the synergistic effects that were observed for certain combinations of ChiA, ChiBand ChiC.

Introduction

Chitin is an insoluble linear polymer of a 1, 4-β-linked polymer of *N*-acetyl-β-D-glucosamine. (GlcNAc), is the second most abundant biopolymer in nature after cellulose, and is a major structure component of the exoskeleton of insects and crustaceans and the cell walls of some fungi; Hydrolysis of the glycosidicbonds in chitin is catalyzed by chitinases, which are found in all types of organisms, from viruses to humans. On the basis of sequence similarities, chitinases are classified into two different families, families 18 and 19, that differ in structure and mechanism of catalysis, The catalytic domains of family 18 chitinases have an $(\alpha/\beta)_8$ (TIM-barrel) fold [9], with an annual production of 100 billon tons [1].

The catalytic $(\beta/\alpha)_8$ barrels of ChiA and ChiBcontain a large insertion between strand 7 and helix7, which makes up a ‘‘wall’’ that lines a deepsubstrate-binding cleft (Fig. 1), in ChiA,this cleft contains six definable subsites (-4 to - 2).The cleft is open on both sidesand it has therefore been suggested that chitin chainscan extend from the active site in both directions andthat the enzyme thus may have some endo-activity.
or more domains that are involved in binding to the substrate (chitin-binding domains) [9]. The soil bacterium *Serratiamarcescens* produces up to In addition to the catalytic domain, many family 18 chitinases contain one four different chitinases (A, B, C1, and C2), a chitin binding protein (CBP21), and a chitobiase when grown on chitin [10].

These enzymes all belong to family 18 chitinases, which have a characteristic catalytic mechanism which depends on participation of the N-acetyl group of the sugar unit bound to the -1 subsite [11]. The crystal structures of ChiA and ChiB have been determined. Based on structural considerations and the outcomes of a variety of experiments, it has been suggested that ChiA and ChiB are exoenzymes that degrade chitin chains from opposite directions [9].

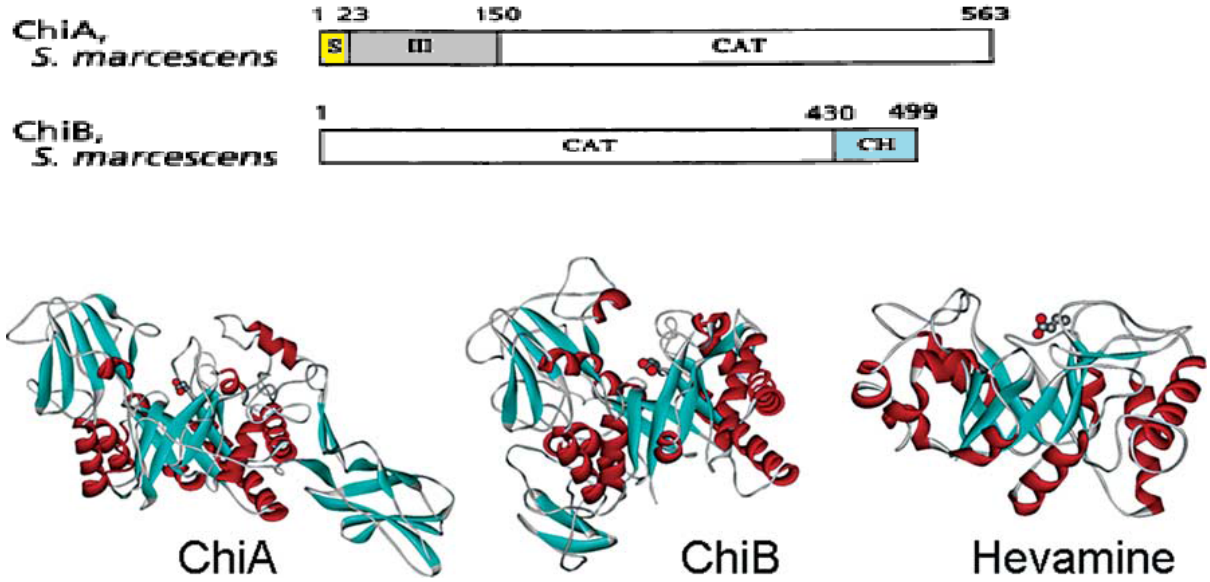

Figure. 1. Domain organization of S. marcescenschitinases and structures of ChiA, ChiB and hevamine. Cat, Catalytic domain; CBM, Carbohydrate Binding Module [3]; FnIII, Fibronectin type 3-like domain; S, Signal sequence. The structures are shown in similar orientations, with the active site cleft/tunnel perpendicular to the paper plane. The side chain of the glutamic acid residue that acts as the catalytic acid/base is show as ball-stick model [10].

Chitinases and chitosanases are capable of converting chitin and chitosans to low molecular mass products (oligosaccharides) by hydrolyzing theβ(1→4) glycosidic linkages between the sugar units. There are several families of chitinases (glycoside hydrolase families 18 and 19) and chitosanases (glycoside hydrolase families 46, 75 and 80. These enzymes differ with respect to their preference for A- and D-units on each side of the scissile glycosidic bond and, consequently, differ with respect to their activities towards different types of chitosan [11].

Individual roles of the chitinases from *Serratiamarcescens* 2170, chitinases A, B, and C1 (ChiA, ChiB, and ChiC1) were produced by Escherichia coli and their enzymatic properties. All three chitinases showed a broad pH optimum and maintained significant chitinolytic activity between pH 4 and 10.
ChiA was the most active enzyme toward insoluble chitins, but ChiC1 was the most active toward soluble chitin derivatives among the three chitinases. Although all three chitinases released $(GlcNAc)_2$ almost exclusively from colloidal chitin, ChiB and ChiC1 split $(GlcNAc)_6$ to $(GlcNAc)_3$, while ChiA exclusively generated $(GlcNAc)_2$ and $(GlcNAc)_4$. Clear synergism on the hydrolysis of powdered chitin was observed in the combination between ChiA and either ChiB or ChiC, and the sites attacked by ChiA on the substrate are suggested to be different from those by either ChiB or ChiC1.Chitinases (EC 3.2.1.14) are the enzymes that hydrolyze β-1, 4-linkages in chitin, one of the most abundant biopolymers in nature

In the classification system of glycosyl hydrolases based on amino acid sequence similarity, chitinases are classified into two different families, 18 and 19. Family 18 chitinases are found in various organisms such as bacteria, fungi, viruses, animals, and higher plants. On the other hand, family 19 chitinases were first found in higher plants and later found in many Streptomyces species as well as a limited number of other bacteria [4].

Materials and methods

Chemicals.Chemicals and Chito-oligosaccharides including alpha chitin (powdered crab shell), were provided by Sigma (St Louis, USA). Allosamidin was provided by S. Sakuda [5]. Bacterial *Serratiamarcecens* (BJL200)was grown in a medium Luria-Bertani (LB) at 30^0 C to extract chromosomal DNA. In LB medium supplemented with 50 mg/ml ampicillin for plasmid preparation of *Escherichia coli* cells, containing Chi A and B in plasmid TOPO TA were grown. pMAY1 was constructed by sub-cloning of the 23 kb EcoRI-Sac1 fragment in pGEM-7f(+)Z (Promega) digested with the same enzymes. pMAY2-10 was constructed by cutting pLES3 with Mld.

Isolation of Chitinase A, B and Allosamidin

Wild-type ChiA and ChiB from *Serratiamarcescens*were over expressed in *Escherichia coli* and purified as described elsewhere [2].
Allosamidinhas been provided after its isolation from *Streptomyces sp*. and the purity was controlled by 1H NMR as described elsewhere. Previously, the structure of allosamidin has been verified by both NMR and Crystallography [5]

Purification of Chitinase A and B by Econo-Column (Chitin Beads)

The periplasm is a concentrated gel-like matrix in the space between the inner cytoplasmic membrane and the bacterial outer membrane called the periplasmic space in gram-negative bacteria. It has been found using cryo-electron microscopy, that a much smaller periplasmic space is present in gram-positive bacteria.

The periplasmic extracts of the stationary phase cultures of *E.coli* harbouring plasmid pMAY20-1 was used for the purification of both chitinases (ChiA and ChiB).[6], and E. *coli*harbouring plasmid pMAY2-10 [7], respectively. Plasmid which encoding the chitinase protein with intact activity. The plasmid was introduced into the *Serratiamarcescens*wild-type strain B JL200 by electroporation.
The extracts were prepared by osmotic shock, as described [8]. One-tenth of the volume of theperiplasmic extracts was taken from the original one where the columns and the media of the chromatographic including the equipment were supplied by Bio-Rad Labs.

The purification of ChiA and ChiB has been determined by using an Econo-Column (Chitin Beads) (Bio-Rad, 2.5 cm, ID, 14 cm with a guard-column using peristaltic pump, monitored at $A_{280.}$ In the optimized purification procedure for ChiA and ChiB, the periplasmic extract in (100 mM Sodium phosphate, pH 6.3) was adjusted to 20 mM Acetic Acid, loaded onto the column at flow rate of $3ml.min^{-1}$. Eluted enzymes 1 ml of Chitinase A and Bseparately were collected and analyzed for purity, were verified by SDS-PAGE, and have been estimated.

SDS-PAGE analysis and Molecular weight Estimation

SDS-PAGE analysis of purified crude preplasmatic, Chi A and Chi B fractions were loaded on ready NuPAGE Gel 10%; at room temperature which using buffer 1X NuPAGE SDS (MOPS), with Bench Marker Protein. Standard molecular weights Bench Marker Proteins thatused purchased from Pharmacia Biotech.

Results

*Purification of chitinase.*The culture of *Serratiamarcecens* filtrate was obtained from the culture broth bycentrifugation and the supernatant was injected by continuous steps to the columns as it has been earlier. The active fractions of chitinase were separated by using Econo-Column, Chitin Beads (Fig. 2).

Chitinase A and Bafter collection and analysis for purity and were verification by SDS-PAGE has been estimated. Two peaks of (Chi A and Chi B) were found to have chitinase activity and pure.
The peaks enzyme were almost completely separated from other proteins, and showing a single band on SDS-P AGE (Fig. 3).

This chitinase was chitinase A and B (ChiA, ChiB). The active fractions were pooled and used as the purified enzyme. The molecular weight of both chitinases was estimated by SDS-polyacrylamide gel electrophoresis as has mention in the materials and methods (Table 1).

Table 1.Chitinases*S.marcescens*

SDS-PAGE	Gene (Protein name)	Localization in *S.marcescens*
57-58	chiA (ChiA)	extracellular
52-54	chiB (ChiB)	periplasm/extracellular

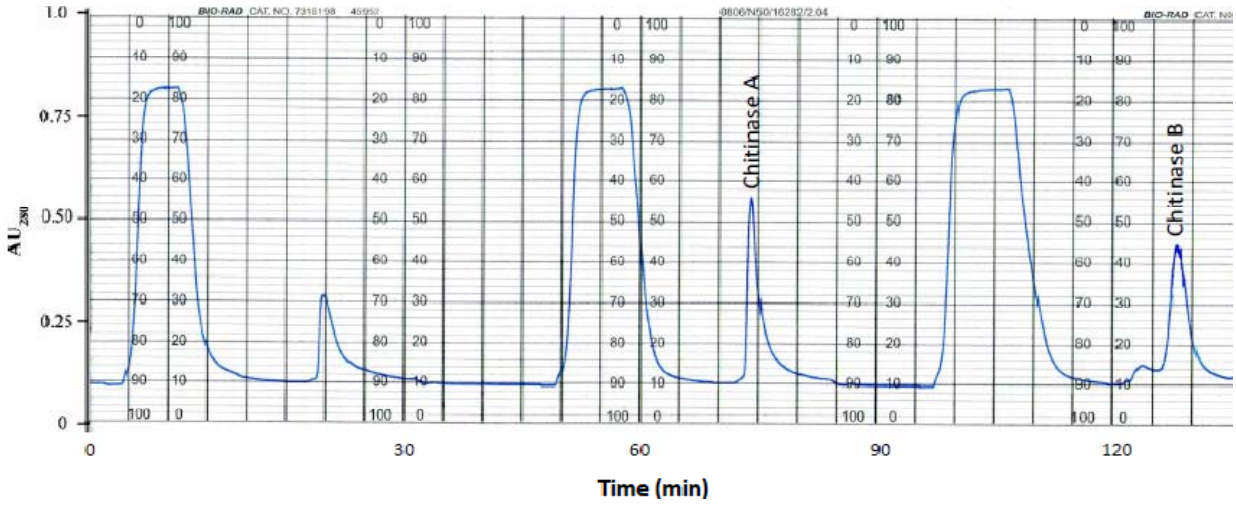

Figure. 2. The purification of ChiA and ChiB has been determined by using an Econo-Column (Chitin Beads) Bio-Rad, 2.5 cm, ID, 14 cm with a guard-column using peristaltic pump, monitored at A_{280}.

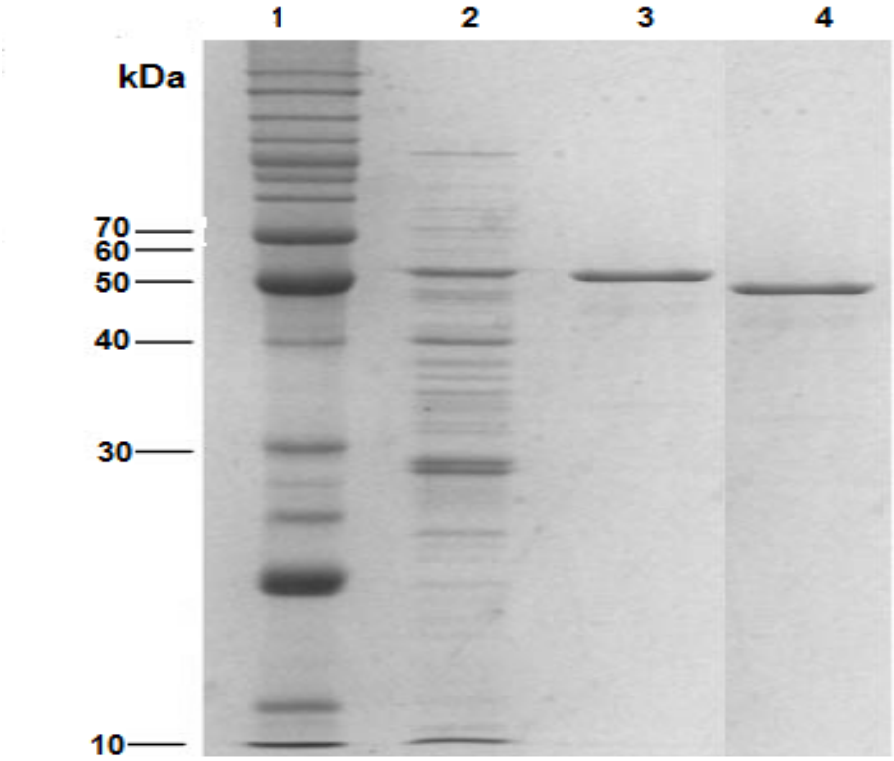

Figure. 3.SDS-PAGE analysis of purified Chi A and Chi B. Lane 1, the approximate positions of Bench marker protein are indicated, lane 2, periplasmic extract of *Serratiamarcecens*, lane 3, purified Chi A: lane 4, purified Chi B.

Discussion

The high stability and many characteristics for the purified Chitinases (ChiA and ChiB) are shared between a broad pH and a temperature optimum between 50^0 C- 60^0 C optimum. The less stable enzyme (ChiB) had the higher optimum temperature for activity.
The optimum temperature for ChiB is probably underestimated since the enzyme is expected to undergo significant thermal inactivation at temperatures near 60^0 C within the time-scale of the activity assay [2].

ChiA which is similar to ChiB for all natural substrates tested when it comes to its product (GlcNAc). For both cases of Chitinases, in ChiB the activity of the endochitinasehydrolasecan causes the formation of monomers in the reaction with chitin which has been confirmed by the detection of (GlcNAc), as products of the reaction with (GlcNAc) as well, in contrast to ChiA.

Several other results support the notion that ChiB possesses a less extended substrate-binding site than ChiA and, consequently, so for chitinaseChiB there will be a possibility of optimization for cleavage of relatively short chito-oligosaccharides. In comparison of both chitinases (ChiA and ChiB), ChiB has a lower specific activity towards chitin than ChiA. Then ChiA can exhibit the cooperative kinetics while is not observed for ChiB.Chitinase (ChiA and ChiB) enzymes seem to have exo-N,N'-diacetylchitobio hydrolase activity , combined with either an endochitinase or an exo-N,N',N'' triacetylchitotriohydrolase activity. In 5**'.***marcescens* ChiA is located extracellularly [2].

In summary, the chitinolytic machinery of *Serratiamarcecens*can ensure translocation of the chitinolytic enzymes to the periplasm and the surrounding medium by involving at least two different export pathways.
The secretion ofchitinaseenzymes that carry standard N terminal signal peptidesother than *Serratiamarcecens*are aremostley known from other bacteria as well. Crystal structures of the one domain family 18 chitinasehevamine (55) and of the multi-domain ChiA from *Serratiamarcecens*were the landmarks

in that family realted to chitinasesenzyems [12]. The catalytic domains of chitinases in family 18 have a TIM-barrel fold as the structures of these enzymes have showed.

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