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Review

The Central Concept for Chitin Catabolic Cascade in Marine Bacterium, *Vibrios*

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Abstract: The enzymatic hydrolysis of chitin has been studied for almost a century, and early work established that at least two enzymes are required, a chitinase that mainly yields the disaccharide *N,N'*-diacetylchitobiose, or $(GlcNAc)_2$, and a "chitobiase", or *β-N*-acetylglucosaminidase, which gives the final product GlcNAc. This pathway has not been completely identified but has remained the central concept for the chitin catabolism through the 20th century[1] including in marine bacteria.[2] However, the chitin catabolic cascade is quite complex, as described in this review. This report describes three biologically functional genes involved in the chitin catabolic cascade of *Vibrios* in an attempt to better understand the metabolic pathway of chitin.

Keywords: chitin catabolic pathway, *Vibrios*, glucosamine-specific kinase, *N,N'*-diacetylchitobiose phosphorylase and *β*-glucosidase.

Role of Marine Bacteria in Degradation of Chitin

Chitin, the linear polymer of *β*-1,4-linked *N*-acetylglucosamine residues, is one of the most abundant organic compounds in nature, with greater than 10^{11} tons produced annually in the marine biosphere alone. Also, chitin is very important component of both the carbon and nitrogen cycles in marine environment. This vast quantity of highly insoluble polymer is degraded so rapidly and is consumed by marine bacteria, such as *Vibrio cholerae* and/or *Vibrio furnissii*, *via* a multitude of tightly regulated genes such as chitinase, *N*-acetylglucosaminidase, deacetylase, chitobioase and so on. Many earlier works showed that marine bacteria play a critical role in converting of this highly insoluble polysaccharides to a biologically and biochemically useful form, and were primarily responsible for this massive turnover.

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Complexity of Chitin Catabolic Pathway

As we described elsewhere, chitin degradation in the marine bacteria is a complex process involving several signal transduction systems and many different classes of enzymes, some with overlapping substrate specificities. Despite chitin catabolism in the marine bacterium *Vibrio furnissii* and *Vibrio cholerae* is much more complex than being expected, involving a minimum of three signal transduction systems and many genes and proteins, only some of which have been identified.[3-12] In this process, extracellular chitin is partially and/or fully hydrolyzed by extracellular chitinase(s), and the oligosaccharides can be diffused through a chitoporin[13] into the periplasmic space. The combined action of two unique enzymes in the periplasm, a chitodextrinase[9] and a specific β-*N*-acetylglucosaminidase,[10] yields two products, GlcNAc and $(GlcNAc)_2$. Particulary, the monosaccharide is taken up *via* the phosphotransferase system (PTS),[12] whereas the disaccharide is taken up unchanged.[8] The further catabolism of $(GlcNAc)_2$ is the major subject to address in-depth the whole pathway of the chitin degrading mechanism in *Vibrios*.

Isolation of Hypothesized Genes

According to the result of sequencing and comparing the homology on data-bank, there are three of hypothetical genes in the chitin catabolic operon named as cosmid clone, pRhexo, which was isolated from genomic DNA of *Vibro furnissii* that has successfully been characterized. Briefly, these genes were subjected to the molecular analysis. The amplified polymerase chain reaction fragments corresponding to each of gene size were cloned into the *Nde*I (5' end) and *Sac*I or other sites available (3' end) of pET21α. The constructed vectors were transformed into the T7 polymerase inducible host strain BL21 (DE3) for overexpression of the protein products. Each of an overexpressed protein products in *E.coli* were purified using a biological tools following purification step. The purified proteins were showed apparently homogeneous in SDS-PAGE, and were subjected to characterize the enzymatic functions to determine the kinetic properties, respectively. In this review, we describe the brief results obtained from the characterization of hypothetical genes in the chitin catabolic in *Vibrios*.

A Novel Enzyme *N,N'*-Diacetylchitobiose Phosphorylase (ChbP)

Thus far, earlier attempts led to the molecular cloning of the periplasmic β-GlcNAcidase were not successful. Many times we attempted to clone the hypothesized cytoplasmic β-GlcNAcidase but isolated a unique enzyme, a phosphorylase. In our earlier work,[4] crude extracts of *V. furnissii* were shown to hydrolyze *p*-nitrophenyl-β-GlcNAc, and the enzyme

$$(GlcNAc)_2 \underset{ChbP}{\overset{K_{eq} = \sim 1.0}{\rightleftharpoons}} \text{GlcNAc-1-P and GlcNAc}$$

Figure 1. Enzymatic reaction of *N,N'*-diacetylchitobiose phosphorylase and its equilibrium constant.

was assumed to be a typical bacterial chitobiase.[2] Numerous unsuccessful attempts were made to clone this enzyme. Instead, an atypical periplasmic β-*N*-acetylglucosaminidase was identified,[10] which has virtually no activity on $(GlcNAc)_2$ at the pH of sea water. The studies were based on the same approach, an attempt to clone the hypothetical chitobiase. We have, instead, cloned, isolated, and characterized a novel enzyme, a specific $(GlcNAc)_2$ phosphorylase that catalyzes the following reaction (Figure 1).

Substrate Specificity of the ChbP

The phosphorolysis takes place with inversion of anomeric configuration, *i.e.* from the β- to the α-glycosidic bond. Other bacterial disaccharide phosphorylases have been characterized. In the classic work of Abeles and coworkers,[14,15] sucrose phosphorylase was shown to convert sucrose (α-Glc) to Glc-α-1-P and fructose. Anomeric configuration is retained by a double displacement mechanism with Glc covalently bound to the enzyme as the intermediate. Other known phosphorylases are all of the single displacement type and are reviewed by Kitaoka *et al.*.[16] Single displacement results in inversion of configuration and the known examples are (all phosphorylases): cellobiose, $\beta \rightarrow \alpha$; maltose, $\alpha \rightarrow \beta$; trehalose, $\alpha \rightarrow \beta$; and laminaribiose, $\beta \rightarrow \alpha$. Thus, the enzyme described here falls within the major group of phosphorylases, catalyzing the phorphorolysis of *N,N'*-diacetylchitobiose, resulting in the inversion of anomeric configuration ($\beta \rightarrow \alpha$).

On the other hand, the reaction catalyzed by ChbP was examined in the reverse direction using GlcNAc-1-P prepared as described above. The reaction was monitored by measuring the rate of P_i formation as it is released from the donor compound, GlcNAc-α-1-P. Reaction conditions such as temperature and pH optima were determined as described for the forward reaction. The enzymatic reaction was characterized as shown in Figure 1.

Molecular Cloning of *gspK* and *bglA* from *V. cholerae*

Earlier study described the cloning of a periplasmic β-*N*-acetylhexosaminidase gene (*exoI*)[10] and an *N,N'*-diacetylchitobiose phosphorylase gene *chbP*[17] from *V. furnissii*. Both *exoI* and *chbP* are critical in the chitin degradation cascade in *Vibrios*. Sequence analysis of the cloned *exoI* from *V. furnissii* suggested that there were two putative open reading

frames (0.8 and 1.7 kb) upstream of the gene. Since many of the chitin catabolic genes that we have cloned from *V. furnissii* are also present in the genome of *V. cholerae*, subsequent studies on the two open reading frames, designated *gspK* (0.8 kb) and *bglA* (1.7 kb), were conducted with *V. cholerae*, respectively.

Enzymatic Kinetics of GspK, A Specific-Glucosamine Kinase

The properties of a glucosamine-specific kinase (MW= ~35 kDa), a recombinant protein overexpressed in *E.coli* and purified as described elsewhere, are described to elucidate the enzymatic kinetics. The enzyme catalyzes the reaction shown in Figure 2. Optimum condition for the enzymatic reaction has been established prior to determine the substrate specificity. Several methods have been used for measuring the rate of sugar-P synthesized by specific kinases and ATP, or, by the phosphoenolpyruvate:glycose phosphotransferase system (PTS). These include the use of [γ-^{32}P]ATP or [^{32}P]P-enolpyruvate or ^{3}H- or ^{14}C-labeled sugar. The labeled hexose-P is separated from the labeled substrates by paper electrophoresis or TLC or small ion-exchange columns (data not shown). A spectrophotometric two-step assay instead was employed to measure ADP because of the availability, stability, and sensitivity of a Cary Bio 100 Varian spectrophotometer kindly made available for our use by Dr. Ernesto Freire (Dept. of Biology, The Johns Hopkins University). This type of assay has been used frequently for measuring sugar kinase activities.

Briefly, GlcN, ATP, and Mg^{2+} were incubated with the purified enzyme in the first step of assay, and the reaction was stopped by heating for 5 min at 100 °C. In the second step, the quantity of ADP produced by the kinase was determined with P-enolpyruvate, pyruvate kinase, NADH, and lactate dehydrogenase. GlcN was omitted from controls to correct for any ADP formed from contaminating enzymes, such as ATPase. The slopes were found to be proportional to the quantity of ADP in the reaction mixture in the range of 10-100 nmol per incubation. ADP formation was also proportional to incubation time and the quantity of protein used in the first step of the assay, and was glucosamine-depend-ent. That is, the formation of ADP in the absence of glucosamine was negligible relative to ADP formed in the complete kinase reaction mixture, even when crude extracts were assayed, probably because the kinase constitutes about 5% of the total protein when it is overexpressed.

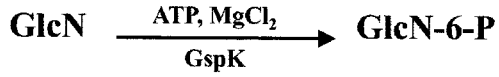

Figure 2. Enzymatic reaction of glucosamine-specific kinase.

Substrate Specificity of the Purified Enzyme

Upon optimizing the reaction condition, we investigated the substrate specificity of the enzyme, GspK. The following sugars tested to determine the substrate specificity of GspK were inactive in assays conducted as long as 12 h: galactosamine, mannosamine, glucose, GlcNAc, $(GlcNAc)_n$, n=2-3, $(GlcN)_2$, lactose, sucrose maltose, and 2-deoxyglucose, galactose and mannose.

Determination of Concentration of GlcN

Neither GalN added to the incubation nor a hydrolysate of crude seagull egg white interfered with the assay, and the recovery of GlcN from the egg white proteins was excellent by comparison with an independent method (data not shown). The standard assay conditions used can determine 10 nmol of GlcN but are easily modified for much greater sensitivity. For instance, the first step in the coupled assay, in which the ADP is generated, comprises a total volume of 0.1 mL, and in the second step, in which the ADP is measured, the volume is 1.0 mL. In the standard assay, only 10 of 100 μL of the first incubation are added to the second, and this can be increased. Secondly, each of the incubation volumes could be reduced 5-fold or more, which would give a 25-fold increase in sensitivity.

A Unique β-Glucosidase from *Vibrio cholerae*

A unique gene, named as cellodextrinase designated as "C" on representative figure, *bglA* was molecularly cloned into *Escherichia coli*, and the protein BglA was overexpressed and purified to apparent homogeneity. BglA is 65 kDa (574 amino acids) with an *N*-terminal amino acid sequence predicted by the gene sequence, suggesting that the enzyme is cytoplasmic.

Substrate Specificity of BglA

The enzyme catalyzes the reaction shown in Figure 3. Kinetic parameters for *p*NP-glucoside were calculated from Woolf-Augustinsson-Hofstee (*v* versus *v*/[*S*]) plots.[18] The kinetic constants were found to be $K_m = 0.45$ mM and $V_{max} = 0.34$ nmol/min/μg. Similar studies with natural substrate cellobiose showed again that the rate of hydrolysis was constant with time of incubation and proportional to protein concentration. The calculated kinetic parameters for cellobiose were $K_m = 6.96$ mM and $V_{max} = 1.60$ nmol/min/μg. Importantly, BglA displayed no activity toward any

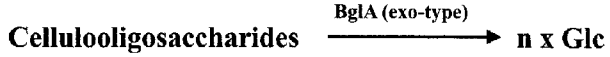

Figure 3. Enzymatic reaction of β-glucosidase.

other α or β sugar substrate tested including di(*N*-acetylglucosamine) $(GlcNAc)_2$, chitobiose $(GlcN)_2$, chitin oligosaccharides $(GlcNAc)_n$ (n = 3~5), trehalose, lactose, maltose, and sucrose. More importantly, it exhibited no activity with positional isomers of cellobiose: sophorose (β1→2), laminaribiose (β1→3), and gentiobiose (β1→6). No activity was detected with *p*NP-β-GalNAc, *p*NP-β-glucose-6-phosphate, or with *p*NP-$(GlcNAc)_n$ (n = 1-3).

Furthermore, BglA was active with the higher β1→4 Glc-linked oligosaccharides, cellotriose, cellotetraose, and cellopentaose. At 1 mM concentrations, pH 6.5, at 37 °C, the rate of Glc formation from these oligomers was around the same as that observed with cellobiose. Due to the enzyme specificity, it has been identified as a unique exo-type; β-glucosidase.

Schematic Draw of the Chitin Catabolic Cascade in *Vibrios*

To briefly summarize: Chitin oligosaccharides, $(GlcNAc)_n$, enter the periplasmic space *via* a specific porin[13] designated 1 in the figure. The smaller sizes of monosaccharides and $(GlcNAc)_2$ allow them to penetrate the cell envelope through constitutive porins in the figure. In the periplasmic space, $(GlcNAc)_n$ oligomers are converted by two unique enzymes, 2 and 3, a chitodextrinase and a β-*N*-acetylglucosaminidase,[9,10] to two products, GlcNAc and $(GlcNAc)_2$. The monosaccharide, GlcNAc, is taken up by the phosphoenolpyruvate:glycose phosphotransferase system,[19,20] specifically by Enzyme II^{Nag}. The gene has been cloned from *V. furnissii*, and the protein has been characterized.[12] The overall reaction of the transport process is: $P\text{-enolpyruvate}_{in} + GlcNAc_{out} \rightarrow GlcNAc\text{-}6\text{-}P_{in} + pyruvate_{in}$. The further metabolism of GlcNAc-6-P involves two steps, 8, a deacetylase, and 9, a deaminase,[21-23] yielding fructose-6-P, NH_3, and acetate. The *nag* regulon of *E. coli*, containing the relevant structural and regulatory genes, has been extensively studied by Plumbridge.[24-26] There are two other possible sources for the key intermediate, GlcNAc-6-P. In *V. furnissii*, $(GlcNAc)_2$ generated both outside the cell by chitinases and in the periplasm from higher oligosaccharides is taken up unchanged by a specific transporter.[8] In the cell, the disaccharide is cleaved by 5, a specific phosphorylase,[17] yielding GlcNAc-1-P and GlcNAc. Consequently, we presume that the GlcNAc-1-P is converted to the 6-P by a specific mutase, 6, known to occur in Neurospora[27] and other cell types.[28,29] The third source of GlcNAc-6-P is a GlcNAc-specific ATP-dependent kinase[30] that is found in *V. furnissii*.[4] The free GlcNAc generated from the disaccharide by the phosphorylase is converted to GlcNAc-6-P by this kinase, 7.

Up to date, as described above it has been known as a routine and/or common sense on chitin degrading mechanisms in marine bacterium *Vibrio furnissii* or *V. cholerae*, until we discovered more unique enzymes involving in chitin catabolic cascade. Upon characterizing the kinetics of unique enzymes, we could put these functional enzymes into the mechanism of chitin degradation in *Vibrios*.

Discussion

Despite the chitin catabolic cascade in *Vibrios* is well established, the original sources of GlcN and cellulooligosaccharides are not identified yet, as shown in Figure 4. The free GlcN is converted to GlcN-6-P by glucosamine-specific kinase,[31] GspK, 10, and also cellulooligosaccharides derived from unknown origin source are converted to glucose by exo-β-glucosidase,[32] 11 that is found in *V. cholerae*. Consequently, the substrate specificity of both BglA and GspK raises an important question. What is the physiological function of these enzymes, and why are they in the cluster of genes we presume to be a $(GlcNAc)_2$ transport and / or chitin catabolic operon?

We can only speculate. To begin with, the same gene is present in the genome of *V. furnissii*. However, neither *V. furnissii* nor *V. cholerae* catabolizes cellobiose or cellulose in contrast to their rapid utilization of chitin oligosaccharides and, more slowly, of chitin. There is at least one possibility that we can suggest. In plants, lignin is a polymerization product of the phenols formed from phenolic β-glucosides such as coniferin and syringin. The glucosides are cleaved

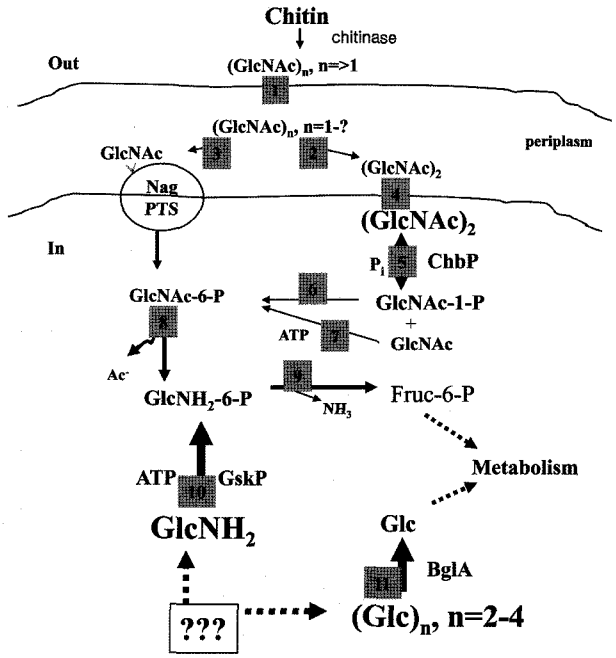

Figure 4. Schematic pathways for metabolism of GlcNAc oligosaccharides. 1. porin, 2. chitodextrinase, 3. β-*N*-acetylglucosaminidase, 4. specific transporter, 5. *N,N'*-diacetylchitobiose phosphorylase, 6. speficic mutase, 7. ATP-dependent GlcNAc-specific kinase, 8. deacetylase, 9. deaminase, 10. glucosamin-specific kinase, 11. β-glucosidase.

by essential plant aryl-β-glucosidases.[33] But the glucoside is first processed by the bacterial β-glucosidase to yield the phenol, coniferyl alcohol, the true inducer of the virulence genes.[34] In other words, the β-glucosidase serves as part of an important signaling sequence in chitin catabolic cascade of *Vibrios*. Because *V. cholerae* spends part of its life cycle associated with marine plants, it is conceivable that BglA has a similar to an enzyme although unknown function, namely to aryl-β-glucosides. Also, the identification of a glucosamine-specific kinase in *V. cholerae* leads to an important question. What function does it serve *in vivo*? The enzyme is cytoplasmic, but what is the origin of free GlcN in the cytoplasm? (Figure 4). Yet remain to be study.

In conclusion, despite we described in this review, three unique genes are cloned and successfully characterized kinetically *in vitro* additionally, still it is so complicated to make figure out the whole structure of the map of chitin degradation mechanism in *Vibrios*. Consequently, the physiological functions of these proteins *in vivo* on chitin catabolic mechanisms of *Vibrios* are not well understood yet, further studies to define the functions of BglA, GspK, and ChbP by constructing an in-frame deletion of the genes in the *V. furnissii* and/or *V. cholerae* genome have to be done, which may result in a significant change in the phenotype of the cells.

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