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Combining chitinase C and *N*-acetylhexosaminidase from *Streptomyces coelicolor* A3(2) provides an efficient way to synthesize *N*-acetylglucosamine from crystalline chitin

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ABSTRACT

The enzymatic bioconversion of chitin is of considerable interest for the natural production of bioactive compounds such as chitooligosaccharides and *N*-acetyl-D-glucosamine (GlcNAc). Key enzymes are involved in the natural processing of chitin, hydrolyzing this abundant biopolymer to yield chitooligosaccharides with substantial value to the medicinal and biotechnological fields. In this study, chitinase C (*Sc*ChiC) from the soil bacterium and chitin decomposer *Streptomyces coelicolor* A3(2) was expressed, purified and characterized. We also optimized a *Streptomyces lividans* system generating *Sc*ChiC expression yields nearly 500-fold higher than the previously reported heterologous expression in *Escherichia coli*. The purified enzyme was found to be stable below 55 °C for a broad range of pH values (pH 3.5–9) and exhibited high activity against chitin and chitooligosaccharides to form chitobiose (C2) as main product. Crab shell chitin hydrolysis profiles also revealed that *Sc*ChiC catalyzes the bioconversion of chitopolysaccharides through an endo-nonprocessive mode of action. When combining *Sc*ChiC with an *N*-acetylhexosaminidase from *S. coelicolor* A3(2) (*Sc*HEX) in an assay using crude extracts and crystalline chitin as substrate, GlcNAc was generated as final product with a yield over 90% after 8 h incubation. This chitin hydrolysis yield represents one of the most efficient enzyme bioconversion of chitopolysaccharides to GlcNAc characterized to date, making the *S. coelicolor Sc*ChiC–*Sc*HEX pair a potentially suitable contender for the viable industrial production of this important bioactive compound.

1. Introduction

Chitin is formed of β(1,4)-linked units of *N*-acetyl-D-glucosamine (GlcNAc), shaping one of the most abundant natural biopolymers on earth. The enzymatic bioconversion of chitin is of considerable interest due to its contribution to the carbon and nitrogen cycles of the biosphere, allowing the transformation of biomass to generate the bioactive GlcNAc compound, a monosaccharide derivative of glucose with substantial industrial value in the medicinal and biotechnological fields (Aam et al., 2010; Chen et al., 2010; Howard et al., 2003). Among other applications, GlcNAc has been used as a supplement for the treatment of osteoarthritis and inflammatory bowel disease (Salvatore et al., 2000; Talent and Gracy, 1996). It is also used in many treatment trials, namely for cancer, autoimmune reactions, sexual disorders, and intestinal diseases (Chen et al., 2010). As a result, the industrial bioproduction of GlcNAc with satisfactory yields and purity remains an imperative for profitable commercialization.

Like cellulose, chitin is insoluble in water. Its hydrogen-bonding patterns require solubilizing agents that induce inter-chain repulsions or disturb intermolecular hydrogen bonding for dissolution. Chitin can be structurally classified into α-chitin—in which the chains of the unit cell are antiparallel – or β-chitin—in which the neighboring chains are aligned in a parallel fashion. The least

Abbreviations: ChiC, chitinase C; *Sc*ChiC, chitinase C from *S. coelicolor*; HEX, *N*-acetylhexosaminidase; *Sc*HEX, *N*-acetylhexosaminidase from *S. coelicolor* A3(2); GlcNAc, *N*-acetyl-D-glucosamine; C2, chitobiose; C3, chitotriose; C4, chitotetraose; C5, chitopentaose; C6, chitohexaose.

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common form of chitin, γ-chitin, is a mixture of parallel and antiparallel chain packing. The different packing patterns of the chitin polymorphs are responsible for their various physicochemical properties. α-Chitin is insoluble in water and the most tightly packed crystalline structure. On the other hand, β-chitin and γ-chitin are less tightly packed, yielding flexible structures that are more water soluble (Cohen, 2010). Every year, more than 100 billion tons of chitin accumulate in nature, making this natural polymer an unlimited and still relatively unexploited biomass resource for the production of GlcNAc (Cohen, 2010; Howard et al., 2003; Kurita, 2006; Tharanathan and Kittur, 2003). Due to extreme insolubility, the large-scale production of GlcNAc from chitin is still far from optimized. Production currently relies on a chemical hydrolysis method that requires concentrated hydrochloric acid at high temperatures (Chen et al., 2010). This process generates a number of industrial and environmental issues, including acidic wastes, low production yields, high energetic costs, and product separation difficulties. In recent years, the environmentally friendly enzymatic processes developed to breakdown chitin have gained in efficiency and robustness, though yields and issues pertaining to final product purity remain challenging (Dai et al., 2011; Jamialahmadi et al., 2011; Nagpure and Gupta, 2013; Pichyangkura et al., 2002; Sashiwa et al., 2002, 2003; Suresh, 2012). As a result, uncovering new and effective biocatalysts and methodologies to optimize chitin degradation remains crucial for the efficient enzymatic production of GlcNAc from this insoluble polymer.

Chitinases, which hydrolyze the β(1,4) linkages of chitin, are key enzymes in the natural processing of this biomaterial. Based on their hydrolytic function, chitinases are classified into endochitinases (E.C. 3.2.1.14), exochitinases (E.C. 3.2.1.29), and *N*-acetylglucosaminidases (E.C. 3.2.1.30) (Cohen-Kupiec and Chet, 1998). The first group randomly cleaves the chitin polymer to yield soluble, low molecular weight oligomers such as chitotetraose (C4) and chitopentaose (C5). The second group catalyzes the progressive release of chitobiose (C2) from the non-reducing or reducing end of the polymer microfibril. Finally, the third group releases *N*-acetylglucosamine monomers (GlcNAc) from chitooligomers and C2, which are produced by the endochitinases and exochitinases, respectively. These enzymes are expected to act in concert to generate complete hydrolysis of the polymer (Cohen, 2010).

Streptomyces spp. are soil bacteria and major decomposers of chitin. *Streptomyces coelicolor* A3(2) is a Gram-positive bacterium whose genome was fully sequenced in 2002, revealing 13 putative chitinases (Bentley et al., 2002). A number of *S. coelicolor* A3(2) chitinase sequences and activities were previously investigated and characterized against chitinous substrates or 4-methyl-umbelliferyl *N,N′,N″*-triacetyl chitotriose (C3), including eight chitinases: ChiA, ChiB, ChiC, ChiD, ChiE, ChiF, ChiG, and ChiH (Kawase et al., 2006; Redenbach et al., 1996; Saito et al., 1999, 2000). The transcription mechanism governing the regulation of these genes and the expression levels of each chitinase were thoroughly investigated (Kawase et al., 2006; Redenbach et al., 1996; Saito et al., 1999, 2000). However, the exo- or endo-character of these *Streptomyces* enzymes, the possible occurrence of multiple catalytic mechanisms (processivity), and how each chitinase is involved in chitin breakdown still remains elusive. Among the abovementioned chitinases, it was shown that *Sc*ChiC displays significant activity toward soluble chitinous substrates, exhibiting the highest activity toward crystalline chitins, including powdered prawn shell chitin and chitin microfibrils (Kawase et al., 2006). *Sc*ChiC shows maximum activity at pH 5–6 when acting on insoluble chitin. The enzyme also displays optimal temperature from 50–65 °C, with maximal activity observed at 60 °C.

This enzyme belongs to glycoside hydrolase family 18 (GH18) and contains three domains: a putative substrate-binding domain, a fibronectin type III-like domain, and a catalytic domain similar to many other GH18 chitinases, such as Chi63 from *Streptomyces plicatus* and ChiIII from *Streptomyces griseus* (Fujii and Miyashita, 1993; Robbins et al., 1992). Although members of this family are evolutionarily diverse, their catalytic domain retains the typical $(\alpha/\beta)_8$ fold. *Sc*ChiC also displays a deep catalytic cleft formed by two insertion domains in the $(\alpha/\beta)_8$ barrel, similar to other chitinases showing high activity on crystalline chitin, such as chitinase A1 from *Bacillus circulans* and chitinase A from *Serratia marcescens* (Kawase et al., 2006). Due to the abovementioned properties, it was suggested that *Sc*ChiC might play a key role in the degradation and utilization of chitin by *S. coelicolor* A3(2) (Kawase et al., 2006).

The GH18 family contains non-processive endo-acting chitinases, as well as processive enzymes with exo- or endo-binding modes. To effectively break down crystalline polymers, non-processive endo-acting enzymes need the presence of substrate-binding and substrate-disrupting domains to increase substrate accessibility (Suzuki et al., 1999; Watanabe et al., 1994). Meanwhile, processive chitinases have the ability to attach to their crystalline substrate during hydrolytic reactions, *i.e.* the substrate successively binds to the active site after each cleavage and slides along the cleft for the next turnover to occur. This processive mechanism is thought to be the most effective way to enzymatically breakdown recalcitrant polysaccharides (Davies and Henrissat, 1995; Horn et al., 2006b; Hult et al., 2005; Sikorski et al., 2006; von Ossowski et al., 2003).

It was reported that the complete degradation of chitin requires the concerted action of chitinases and *N*-acetylhexosaminidases (HEX) (Horsch et al., 1997; Seidl, 2008). In this mechanism, chitinases degrade chitin into chitooligomers, which are subsequently hydrolyzed by HEX to release GlcNAc (Saito et al., 2008, 2007). We recently characterized a β-*N*-acetylhexosaminidase from *S. coelicolor* A(3) 2 (*Sc*HEX), an enzyme displaying significant hydrolytic activity toward chitooligosaccharides, from which it can yield pure GlcNAc without any byproduct (Nguyen-Thi et al., 2014). In the present work, we optimized a *S. lividans* recombinant expression system to produce and characterize *Sc*ChiC from *S. coelicolor* A3(2) (gene accession number 2SC6G5.20c). We further clarify the chitinolytic system of this important soil bacterium, in addition to developing and characterizing an efficient enzyme combination mixture using *Sc*ChiC and *Sc*HEX to help improve the enzymatic breakdown of raw chitinous biomass to produce the industrially-relevant GlcNAc compound. We observe that the *Sc*ChiC–*Sc*HEX pair performs one of the most efficient enzymatic bioconversion of chitooligosaccharides to GlcNAc characterized to date, promising high applicability for raw biomass transformation.

2. Materials and methods

2.1. Gene expression and protein purification

All molecular biology procedures in *Streptomyces* were performed according to standard published methods (Hopwood et al., 1985). The *S. coelicolor* A3(2) gene encoding for *Sc*ChiC (accession number 2SC6G5.20c) was amplified by PCR. *Sph*I and *Sac*I restriction sites (underlined) were designed in forward primer 5′-AAAGCATGCGCTTCAGACACAAAGC-3′ and reverse primer 5′-TTTGAGCTCTTACTTGAGGCCGCTGTC-3′. The PCR product was inserted into the *Sac*I-*Sph*I-digested pC109 plasmid (derived from pIJ702) (Hurtubise et al., 1995), which carries a thiostrepton resistance marker. The resulting plasmid was transformed into *Streptomyces lividans* 10–164 [*msiK*$^-$] protoplasts (Hurtubise et al., 1995) and plated on R5 medium without antibiotic at 34 °C. After 16 h, the agar plates were flooded with thiostrepton solution and further incubated for 3–4 days at 34 °C. The resulting vector construct was confirmed by DNA sequencing and further employed