Characterization of an exo – ß – D - glucosaminidase from the hyperthermophilic archaeon *Pyrococcus furiosus*

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Abastract. D-glucosamine is an important chemical compound in industry. Exo – ß – D - glucosaminidase (EC number: 3 .2 .1 .165) is a potential enzyme which can be used in production of D-glucosamine. In this study, an exo-ß-D-glucosaminidase from the hyperthermophilic archaeon *Pyrococcus furiosus* was cloned and expressed in *E. coli* and purified to homogeneity. The therrnostable enzyme had a moleculat mass of 90 kDa and could hydrolyze chitosan or chitooligosaccharide to produce D-glucosamine. Moreover, thin layer chromatography showed that the enzyme could not act on lactose, indicating it is not a ß-galactosidase. The enzymatic properties of this exo-ß-D-glucosaminidase suggest its potential applications in biotechnology to produce D-glucosamine.

Introduction

D-glucosamine hydrochloride (GlcN), which is a hydrolyzed product of the acetylated polysaccharide chitin, is widely used in medicine, food, feedingstuff and makeup industry. It is a precursor of the anticancer drug chlorozotocin and plays important roles in the treatment of chondropathy [1]. Nowdays, China is the most GlcN production country in the world, and the GlcN industry has reached up to billons of dollars every year.

In industry, GlcN is generally produced by concentrated hydrochloric acid hydrolysis of chitin-the insoluble natural macromolecule from the shell of shrimp and crab, followed by concentration, decoloration and crystallization. However, the severely environmental pollution resulted from this production procedure has greatly restricted the sustainable development of GlcN industry, namely, green and environment-friendly manufacturing [2].

Compared with the chemical engineering, bioengineering such as fermentation, enzyme catalysis has the advantages of safety, less pollution, reduced end-products and easy to control etc [3]. For example, the method in production of chitooligosaccharide by chitosanase catalysis has been widely used [4,5]. Hence, exploring the effective and environment-friendly GlcN production method in biotechnology is important for the healthy progress of chitin industry.

GlcN is the monomer of chitosan or chitooligosaccharide, which can be catalytically produced by exo-ß-D-glucosaminidase (EC number: 3.2.1.165). Exo-ß-D-glucosaminidase has been reported from several microorganisms [6-7]. In this study, we report the characterization of an exo-ß-D-glucosaminidase from the hyperthermophilic archaeon *Pyrococcus furiosus*, which catalyzes chitosan or chitooligosaccharide to produce GlcN.

Materials and Methods

The *P. furiosus* genome was presented by Dr Xipeng Liu (Shanghai Jiaotong University, China). Restriction enzymes, *Pfu* DNA polymerase, and the DNA ligation kit (ver.2.1) were from Takara (Dalian, China). The cloning host *Escherichia coli* strain DH5α, expression host BL21(DE3)-CodonPlus-RIL and plasmid pET15b were purchased from Novagen (Madison, WI, USA). Unless stated, all chemicals were obtained from Sangon Biotech (Shanghai, China).

Cloning of the *P. furiosus* exo-ß-D-glucosaminidase gene

To clone the *P. furiosus* exo-ß-D-glucosaminidase gene, two primers [sense (5′- GAC CATAT G G T A A A A CC T A T C T T C C T T G A T G G-3′) and antisense (5′- C A G T C G A C C T A A A C T C T T A T T T C G A A T G T T T C G – 3′), underlined nucleotides indicated the *Nde*I and *Sal*I restriction enzyme sites respectively)] were designed based on the *P. furiosus* genome sequence. The gene was amplified by PCR. The PCR product was digested by *Nde*I and *Sal*I and then cloned into plasmid pET15b. The recombinant plasmid was verified by DNA sequencing.

Expression and purification of the *P. furiosus* exo-ß-D-glucosaminidase

The expression host *E. coli* BL21-CodonPlus (DE3)-RIL was transformed with the recombinant plasmid and cultivated in LB medium at 37°C with 100 μg ml^{-1} ampicillin and 34 μg ml^{-1} chloramphenicol. An overnight culture was diluted 1:100 and grown until the OD_{600} reached 0.5, and then induced with 0.5 mM IPTG at 37 °C for 4 h. To purify the recombinant protein, the crude cell extract was sonicated, heated at 85 °C for 30 min to obtain heat-stable proteins and then centrifuged (12000×g for 30 min). The supernatant was filtered using a 0.22 μm pore-sized membrane filter, loaded onto a nickel column (Novagen), and eluted with 80 mM Tris/HCl (pH 7.9) containing 200 mM imidazole. The purified protein was analyzed by SDS-PAGE and the protein concentration was determined according to the Bradford method [8].

TLC analysis of the enzyme activity and hydrolytic mode

To analyze reaction mode of the recombinant enzyme, 100 ng purified protein was added in the standard reaction mixture containing 1% (w/v) substrates lactose, chitosan and chitooligosaccharide respectively. The reaction was performed at 90 °C for 1 h, and the product was analyzed by silica gel thin layer chromatography (TLC, Merck). n-butanol: ethanol: ammonia: H_2O (5: 4: 2: 1, v/v) was used as developer, and amino saccharides were detected with the ninhydrin reagent.

Results and Discussion

Sequence analysis of the *P. furiosus* exo-ß-D-glucosaminidase

In the *P. furiosus* genome, a gene (PF0363, Genbank: AAL80487.1) was annotated as a ß-galactosidase. It is consisted of 2319 bp and encodes a protein of 772 amino acids with a predicted molecular mass of 90.2 kDa (http://www.expasy.org/tools/pi_tool.html). The deduced amino acids sequence has overall identity to the characterized exo-ß-D–glucosaminidases from the hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1 (63%, BAD85943.1) and *Pyrococcus horikoshii* (83%, BAA29599.1). Furthermore, the enzyme shows high sequence similarity to the putative ß-galactosidases from archaeon *Pyrococcus abyssi* (71%, CAB50440.1), *Pyrococcus sp*. ST04 (72%, AFK22159.1), *Thermococcus nautili* (68%, AHL22957.1) and *Thermococcus sp*. AM4 (65%, EEB74590.1). However, it shows no identity with the reported exo-ß-D-glucosaminidases from Eukaryotes and Bacteria up to now, for example, exo-ß-D-glucosaminidases from fungi *Trichoderma reesei* (BAD99604.1) and bacteria *Amycolatopsis orientalis* (AY962188.1). Conserved domain search indicated that the protein had two regions homologous to the ß-galactosidases in family 35 and 42 glycosyl hydrolases, respectively [9, 10].

Cloning and expression of the *P. furiosus* exo-ß-D-glucosaminidase

The *P. furiosus* exo-ß-D-glucosaminidase gene was cloned into the plasmid pET15b. The recombinant plasmid was verified by DNA sequencing and double restriction enzymes (*Nde*I and *Sal*I) digestion analysis (Fig. 1A). The recombinant protein was over-expressed and purified to homogeneity as described. SDS-PAGE showed a size of about 90 kDa which was in accordance with the calculated molecular mass of 90.2 kDa (Fig. 1B). These results indicate that the *P. furiosus* exo-ß-D-glucosaminidase is correctly expressed and purified in *E. Coli*.

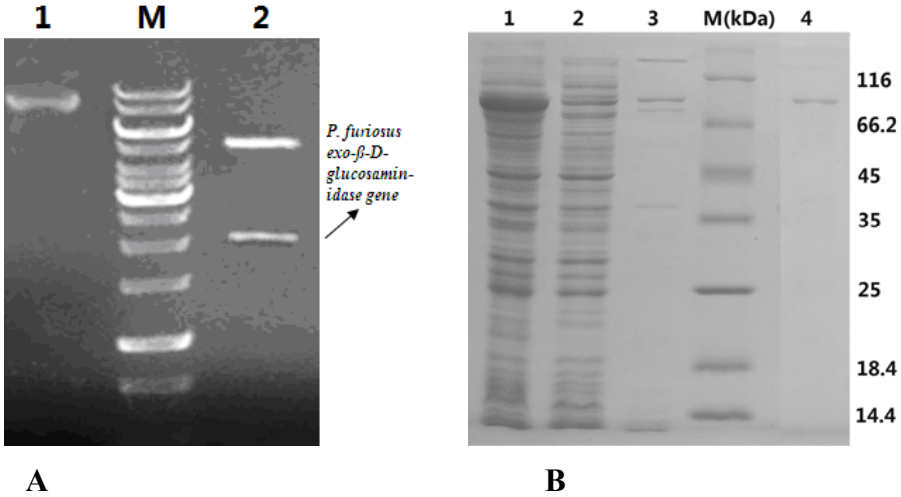

Fig. 1 **A**: Cloning of the *P. furiosus* exo-ß-D-glucosaminidase gene. Lanes: 1. the recombinant plasmid with *P. furiosus* exo-ß-D-glucosaminidase gene; M. DNA markers. From top to bottom: 10000, 8000, 6000, 5000, 4000, 3500, 3000, 2500, 2000, 1500, 1000, 750 bp. 2. double restriction enzymes (*Nde*I and *Sal*I) digested recombinant plasmid. **B**: SDS-PAGE analysis of the *P. furiosus* exo-ß-D-glucosaminidase. Lanes: 1. crude cell extract; 2. soluble fractions; 3. thermostable protein after heat-treatment at 85 °C for 30 min; M. protein molecular markers; 4. the purified protein after Ni^{2+} column affinity chromatography.

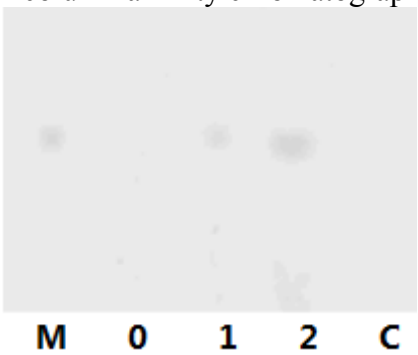

Fig. 2 TLC analysis of the *P. furiosus* exo-ß-D-glucosaminidase activity. The reaction procedures were as described in "Materials and Methods". M. D-glucosamine; 1. reaction for 5 min; 2. reaction for 30 min; 3. reaction for 1 h; C. chitosan without enzyme added.

Enzyme activity and mode of action of *P. furiosus* exo-ß-D-glucosaminidase

TLC showed that the enzyme could hydrolyze the partly deacetylated chitin-chitosan and chitooligosaccharide to produce the end product D-glucosamine (Fig.2). These results verify that the protein encoded by the gene PF0363 (AAL80487.1) is an exo-ß-D-glucosaminidase. Considering the biotechnological advantages of thermophilic enzymes such as avoiding contamination, allowance of higher substrate concentrations and improvement of the rate of reaction, the exo-ß-D-glucosaminidase reported from archaeon in this study thus provides a novel way to produce D-glucosamine in manufacturing efficiently and environment-friendly.

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