Protein Expression and Purification 71 (2010) 108–114

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

# Expression of recombinant endochitinase from the Antarctic bacterium, *Sanguibacter antarcticus* KOPRI 21702 in *Pichia pastoris* by codon optimization

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ARTICLE INFO

*Article history:*
Received 27 October 2009
and in revised form 23 December 2009
Available online 25 January 2010

*Keywords:*
Antarctica
Endochitinases
Codon optimization
Recombinant
*Pichia pastoris*

ABSTRACT

An endochitinase was previously purified and the gene was cloned from the psychrophilic Antarctic bacterium, *Sanguibacter antarcticus* (KCTC 13143). In the present study, recombinant endochitinase, rChi21702, was expressed using a yeast expression system (*Pichia pastoris*) and codon optimization. The expressed rChi21702 was purified by Phenyl-Sepharose column chromatography. Optimal expression yielded 1-mg purified enzyme from 1-L bioreactor culture. When *p*-NP-$(GlcNAc)_2$ was used as a substrate, the specific activity of the enzyme was determined to be 20 U/mg. In vitro assays and thin-layer chromatography demonstrated that the recombinant enzyme has endochitinase activity that produces diacetyl-chitobiose as a dominant end product when chitooligomers, colloidal chitin, and the chromogenic *p*-NP-$(GlcNAc)_2$ are used as substrates. Optimal activity for rChi21702 was observed at 37 °C and a pH of 7.6. Interestingly, rChi21702 exhibited 63% of optimal activity at 10 °C and 44% activity at 0 °C. Taken together, the results indicate that rChi21702 has psychrotolerant endochitinase activity even after recombinant expression in yeast cells.



## Introduction

As one of the most abundant polysaccharides in nature, chitin is regarded as a critical carbon source for many Antarctic bacteria. In previous research, a large amount of chitin was found in penguin *guano* as penguins consume krill for their diet [1,2]. This chitin-rich source contributes to carbon recycling, and, thus, makes up a critical part of Antarctic ecosystems [3]. Chitin is a large family of glycans which is unbranched homopolymer of β-1,4-linked *N*-acetylglucosamine (GlcNAc). It composes the cell walls of some microbes such as fungi, and exoskeletons of invertebrates, including insects, and crustaceans. Chitinases (EC3.2.1.14; 1,4-β-poly-*N*-acetylglucosaminidase) hydrolyze the β-1,4 linkage of chitin [4]. Chitinases, in general, are divided into two categories: endochitinases and exochitinases. Endochitinases (EC3.2.1.14) cleave chitin polymers at random internal sites, whereas exochitinases cleave chitin progressively starting at the non-reducing ends of the chains and release *N*-acetyl-D-glucosamine (GlcNAc) monomers or diacetyl-chitobiose by β-(1,4)-*N*-acetylglucosaminidase activity (EC3.2.1.30) or 1,4-β-chitobiosidase activity (EC3.2.1.29), respectively [5].

The enzymes have been found in diverse organisms. Viral chitinases function in pathogenesis [6]. Bacterial chitinases are known to digest chitin for utilization as a carbon and energy source, and, thus, recycle carbon stored in chitin [7]. In fungi, chitinases play roles in autolytic, nutritional, and morphogenetic functions [8,9]. In insects, chitinases have important roles in post-embryonic development and degradation of old exocuticle in molting [10]. Chitinases in plants are important in defense and development [11]. Chitinases in humans are thought to play a role in defense against pathogens, which have chitinous structures [12]. Exochitinases and endochitinases are considered important enzymes in biomedical industry, and are used to produce GlcNAc and chitooligosaccharides [13].

Bacterial expression systems are the most common and preferred method for the overexpression of recombinant proteins because of several advantages, including fast cell growth, inexpensive media, and relatively simple gene manipulation. Bacterial systems, however, can cause problems such as protein misfolding and inclusion body formation, and thus yield non-functional proteins [14]. Yeast expression systems have the advantage of Eukaryotic protein processing, folding, and post-translational modification machinery [15,16]. In addition, other reports have described recombinant proteins that do not fold correctly in bacterial systems, but are expressed successfully in yeast systems [17,18]. Heterologous protein expression raises another problem of poor protein expression because of biased codon usage. This problem is minimized by improving the genetic code by partly or fully rearranged synthetic codons. Many studies have recently reported successful heterologous protein expression by codon optimization and eliminating secondary structure of mRNA [19–21].

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doi:10.1016/j.pep.2010.01.017

An extracellular chitinase with psychrotolerant characteristics was recently purified by our lab from the Antarctic bacterium, *Sanguibacter antarcticus* KOPRI 21702 (KCTC 13143). We previously obtained the coding sequence for this enzyme from genomic DNA [22]. However, production of the native recombinant endochitinase in either *Escherichia coli* or *Pichia pastoris* was unsuccessful. In the present study, cold-adapted endochitinase chi21702 from Antarctic bacterium was successfully expressed in the methylotrophic yeast, *P. pastoris* by codon optimization.

## Materials and methods

### *Strains, plasmids, media, and materials*

The chitinase genes were expressed in *P. pastoris* X33 (Invitrogen, Carlsburg, CA, USA) using the plasmid vector, pPICZαA (Invitrogen, Carlsburg, CA, USA). *P. pastoris* strains were grown in YPD for general growth. Transformants of *P. pastoris* were selected on YPD medium containing zeocin (100 μg/ml). To induce the chitinase synthesis, *P. pastoris* cells grown in BMGY were transferred into BMMY medium and incubated for 96 h. *S. antarcticus* (KCTC 13143) was grown in Zobell medium and the culture medium was used as wild-type Chi21702 enzyme. Anti-cMyc antibody and anti-6XHis tag antibody were obtained from Abcam PLC (Cambridge, UK). c-Myc tagged Protein mild purification gel was purchased from MBL (Woburn, MA, USA). Restriction enzymes were from Fermentas (Ontario, Canada) and DNA ligation kit (Mighty Mix) was from Takara (Shozo, Kyoto, Japan). All other chemicals were from Sigma, Invitrogen, and Merck (USA).

### *Codon optimization of Chi21702*

To generate mature enzyme, 25 amino acids of putative signal peptide (MRSIRAAALTAALALLAAIPAAGMT) were eliminated and the coding region of the mature chi21702 gene was optimized according to the nuclear codon usage of *Pichia* using proprietary algorithms that replace rare codons, problematic mRNA structure, and various cis-elements in transcription and translation (GenScript, Piscataway, NJ). An *Xho*I restriction site was attached to the 5′ end and an *Xba*I site was added to the 3′ end of the optimized sequence to facilitate the subcloning into yeast expression vector.

### *Construction of expression vectors and preparation of yeast strains*

The rChi21702 enzyme was expressed in the methylotrophic yeast *P. pastoris* using the EasySelect *Pichia* Expression Kit (Invitrogen). The synthetic codon-optimized chi21702 gene was excised by digestion with *Xho*I and *Xba*I and ligated into a yeast pPICZαA expression vector (Invitrogen). The construct was designed to yield the mature 481 amino acid rChi21702 fused in-frame with a C-terminal c-myc epitope and 6XHis tag. The calculated molecular weight of the fusion protein was 53 kDa. Approximately 5 μg of recombinant plasmid was linearized with *Pme*I restriction enzyme and used to transform the X33 strain of *P. pastoris* by electroporation using a MicroPulser (Bio-Rad, Hercules, CA, USA). The genomic DNAs of transformants were used as a PCR template with the yeast expression vector-specific 5′ and 3′ AOX1 primers according to the manufacturer's instructions.

### *Selection of recombinant cells and expression of rChi21702*

Several transformants grown on YPD plate containing zeocin were inoculated onto a colloidal chitin-containing BMMY plate and induced daily by placing with 100% methanol on the lid of the inverted plate. Small scale cultures (25 ml) of cells showing clear zone around the colonies on the agar plate were induced with 0.5% methanol and 1-ml samples were removed at time points ranging from 0 to 96 h. Samples were centrifuged (12,000*g*, 3 min); supernatant and cell fractions were stored at −80 °C until analysis for the presence of expressed rChi21702 protein by Western blot and enzyme assay.

### *Fermentation*

Cultivation of recombinant *P. pastoris* X33 was performed in a 1-L jar fermenter (Biostat Q, B. Braun Biotech International, Melsungen, Germany). Fifty ml of seed containing YPD medium was prepared and incubated with shaking at 25 °C, 200 rpm for 20 h. Fermentation medium consisted of 40 g/l glycerol, 26.7 ml/l $H_3PO_4$, 0.93 g/l $CaSO_4$, 18.2 g/l $K_2SO_4$, 14.9 g/l $MgSO_4{\cdot}7H_2O$, 4.13 g/l KOH, and 4.35 ml/l trace metal solution (6.0 g/l $CuSO_4{\cdot}5H_2O$, 0.08 g/l NaI, 3.0 g/l $MnSO_4{\cdot}H_2O$, 0.2 g/l $NaMO{\cdot}2H_2O$, 0.02 g/l $H_3BO_4$, 0.5 g/l $CoCl_2$, 20.0 g/l $ZnCl_2$, 65.0 g/l $FeSO_4{\cdot}7H_2O$, 0.2 g/l Biotin, and 5 ml/l $H_2SO_4$). Temperature was maintained at 25 °C, and pH was set between 5.0 and 5.5 with 10% (v/v) $NH_4OH$. Cells were grown for 34 h after inoculation in the batch culture until the glycerol was completely exhausted and then DO-stat fed-batch culture was started and carried out for 108 h in methanol feed medium (methanol and 12 ml/l trace metal solution). The DO set-point for DO-stat fed-batch culture was 50% air-saturation and total 72 ml of the feed medium was supplied during fermentation. Growth of the *P. pastoris* was monitored by measuring dry cell weight (DCW[1]).

### *Purification of rChi21702*

Fermentor-cultured medium of the X33 yeast strain transformed with Chi21702-cMyc/His induced with methanol was subjected to 80% ammonium sulfate precipitation, followed by dialysis against 20 mM Tris–Cl, pH 8.0. The dialysate was loaded onto a 30 × 10 mm Phenyl-Sepharose column (GE Healthcare), washed with a buffer containing 1.5% isopropanol and 20 mM Tris–Cl, pH 8.0, and eluted in steps with 1-ml elution buffer containing 2%, 2.5%, and 3% isopropanol.

### *Protein quantification, electrophoresis, and Western blot analysis*

Protein concentration was determined using Bradford reagent. SDS–PAGE was performed on a 12.5% polyacrylamide gel, and the resolved protein bands were stained visualized using Coomassie Brilliant Blue G-250. Proteins were transferred to 0.22 μm PVDF, probed with primary antibody (either chicken anti-cMyc or chicken anti-6XHis at 1:10,000 dilution), and incubated with HRP-conjugated secondary antibody (rabbit anti-chicken antibody, 1:100,000). Immunoreactive bands were visualized using SUPEX Western blot detection reagents (Takara, Kyoto, Japan) and LAS-3000 imaging system (Fujifilm Life Science, Tokyo, Japan).

### *Enzymatic activity assays*

Specific enzyme activity was measured by a colorimetric assay with *p*-nitrophenyl-β-D-*N,N′*-diacetyl-chitobiose [*p*-NP-$(GlcNAc)_2$] as a substrate. The reaction mixture (1 ml), containing 0.1 mM *p*-NP-$(GlcNAc)_2$ and 3 μg rChi21702 was incubated at 37 °C for 15 min. The reaction was terminated by adding 0.2 ml stop solution (0.4 M sodium carbonate buffer, pH 9.6). The amount of

[1] *Abbreviations used:* DCW, dry cell weight; *p*-NP-$(GlcNAc)_2$, *p*-nitrophenyl-β-D-*N,N′*-diacetyl-chitobiose; $(GlcNAc)_2$, *N,N′*-diacetyl-chitobiose; $(GlcNAc)_3$, *N,N′,N″*-triacetylchitotriose; $(GlcNAc)_4$, *N,N′,N″,N‴*-tetraacetylchitotetraose; $(GlcNAc)_5$, penta-*N*-acetylchitopentaose; $(GlcNAc)_6$, hexa-*N*-acetylchitohexaose.

*p*-nitrophenol formed by enzyme reaction was determined by measuring the absorbance at 405 nm and using the extinction coefficient, 17,000 $M^{-1}$ $cm^{-1}$. A unit of activity (1 U) was defined as 1 μmol substrate hydrolyzed to *N*-acetylglucosamine and *p*-nitrophenol/h/L.

### *Thin-layer chromatography (TLC)*

*N*-acetyl-D-glucosamine (GlcNAc), *N,N′*-diacetyl-chitobiose $(GlcNAc)_2$, *N,N′,N″*-triacetylchitotriose $(GlcNAc)_3$, *N,N′,N″,N‴*-tetra-acetylchitotetraose $(GlcNAc)_4$, penta-*N*-acetylchitopentaose $(GlcNAc)_5$, hexa-*N*-acetylchitohexaose $(GlcNAc)_6$, and colloidal chitin were used as substrates. Each substrate [(3 mM GlcNAc, 2 mM $(GlcNAc)_2$, 2 mM $(GlcNAc)_3$, 2 mM $(GlcNAc)_4$, 1.5 mM $(GlcNAc)_5$, and 1.5 mM $(GlcNAc)_6$] was separately incubated with 50 ng rChi21702 at 30 °C in a mixture (0.1 ml) containing 50 mM sodium phosphate buffer, pH 7.6, for 0, 1, 2, or 6 h. Colloidal chitin (800 μg) was used in a similar assay, but with different incubation times (0, 1, 3, and 5 h). The negative controls lacked enzyme. The reaction was terminated by adding 0.2 ml of 0.4 M sodium carbonate buffer, pH 9.6. Aliquots (10 μl) of the reaction mixtures were then chromatographically separated on silica gels 60 $F_{254S}$ (Merck, USA) using a solvent system of 2-propanol:water:ammonia (34:15:1), and the signal was detected by spraying with an aniline-phthalate solution (2 mM aniline and 3.3% phthalic acid in water-saturated butanol) and subsequent heating to 160 °C. Size markers were prepared by combining 4 mM GlcNAc, 2 mM $(GlcNAc)_2$, 2 mM $(GlcNAc)_3$, 2 mM $(GlcNAc)_4$, 1 mM $(GlcNAc)_5$, and 1 mM $(GlcNAc)_6$.

### *Determination of optimal temperature and pH*

Optimal temperature for rChi21702 and native Chi21702 activity was determined by testing the hydrolysis of *p*-NP-$(GlcNAc)_2$ as a substrate, under conditions described above, temperatures tested ranged from 0 to 60 °C for 1 h. For assessing the optimal pH activity, buffers at pH ranging from 4.0 to 9.5 (50 mM sodium acetate buffer for pH 4.0–6.0 and 50 mM potassium phosphate buffer for pH 7.0–9.5) were used. Reactions were carried out using *p*-NP-$(GlcNAc)_2$ at 37 °C for 1 h. All assays were performed in triplicate and the mean values were used to plot each time or pH point.

## Results

### *Codon optimization of chi21702*

The native gene employed tandem rare codons that can reduce the efficiency of translation or even disengage the translational machinery in yeast *Pichia* strains. The sequences of native (GenBank accession #DQ386639) and codon-optimized genes were aligned and this alignment demonstrates that the optimization did not change the amino acid sequence (Fig. 1A). The relative frequency of codon usage was adjusted to the most preferred triplets

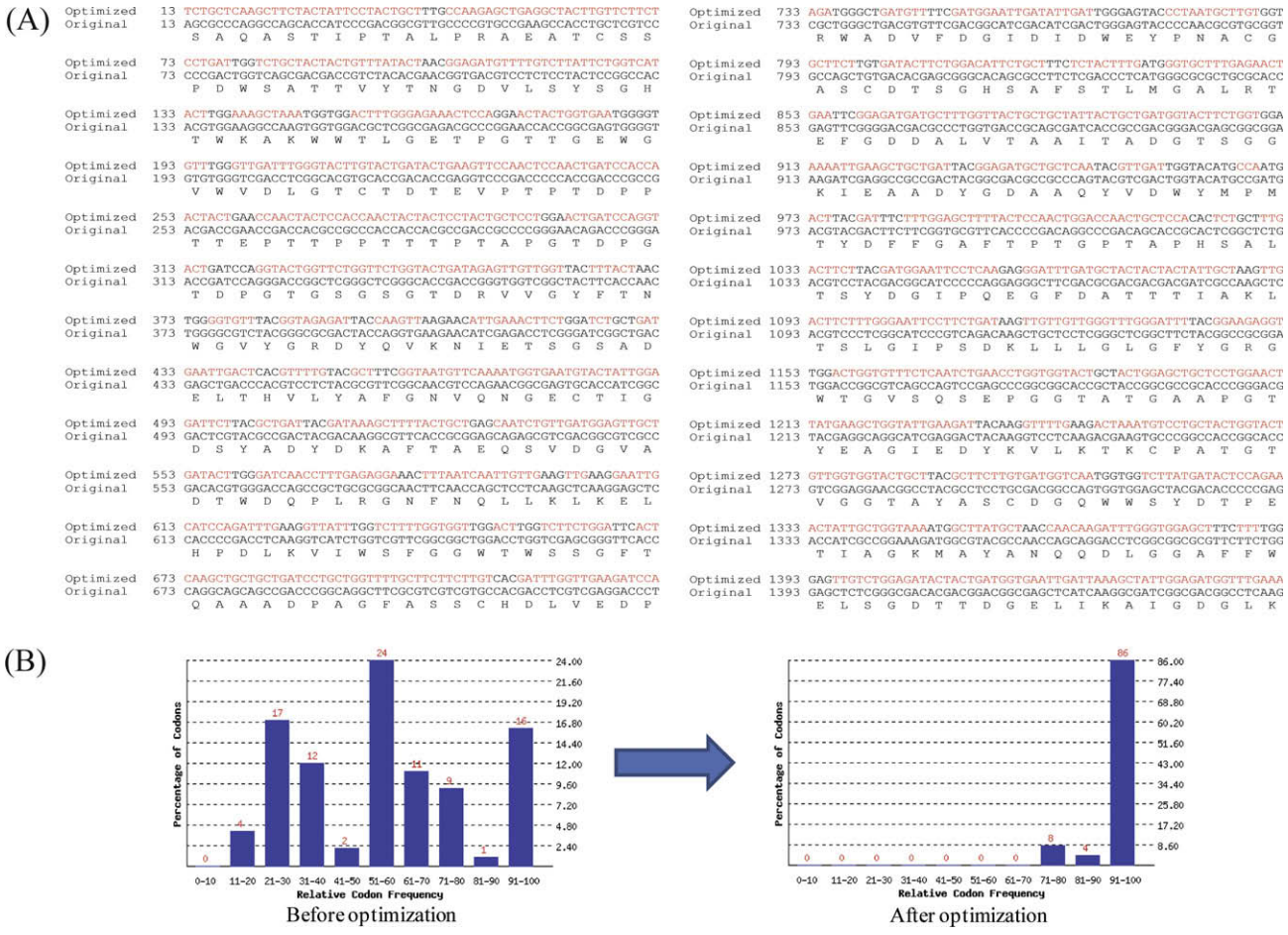


**Fig. 1.** Codon optimization of chi21702 for expression in *Pichia*. (A) Sequence alignment of codon-optimized and wild-type chi21702. Bases modified to optimize codons are in red and wild-type bases are in black. Ultimately, 402 out of 480 codons (almost 84%) were substituted. (B) Enhanced codon usage of chi21702 for expression in *Pichia*. The relative frequencies of individual codons for the native chi21702 gene (GenBank accession #DQ386639) in *Pichia* are indicated in the left panel with the most frequently used triplet in *Pichia* set to 100%.

in *Pichia* strains so that overall codon usage bias in *Pichia* was enhanced after codon optimization (Fig. 1B).

### *Construction of recombinant Pichia strains*

Control cells transformed with pPICZαA vector showed no clear zone around colony boundaries (Fig. 2A, pPICZαA-only). Cells transformed with pPICZαA-chi21702 revealed distinct clear zone around colony boundaries (Fig. 2A, pPICZαA-21702), indicating that the rChi21702 was expressed and secreted.

The yeast strain transformed with the chi21702 plasmid construct was expected to secret mature Chi21702 fused in-frame with a C-terminal c-myc epitope and polyistidine tag. An immunoreactive protein of the predicted mass was detected only in methanol-supplemented culture media containing X33 yeast cells transformed with chi21702 plasmid (data not shown). Neither the GS115 cells nor the control recombinant yeast cells transformed with parent vector lacking a Chi21702 insert secreted an immunoreactive protein when cultured in the presence of 0.5% methanol (data not shown). The cell lines having maximal secretion and chitinase activity were selected and subjected to large scale fermentation in a bioreactor.

### *Chitinase production by feeding methanol in a bioreactor*

Chitinase production by fermentation was divided into two phases: batch and induction fed-batch (Fig. 2B-1 and B-2, respectively). The methanol feed medium was manually added when DO level was above the set-point, and not added below this set-point. The cell density gradually increased and reached the maximum value of 44.4 g/l dry cell at 108 h after induction. However, chitinase activity in the culture broth increased to 30 U/L at 84 h after induction and then slightly decreased to 25 U/L at 108 h after induction (Fig. 2B).

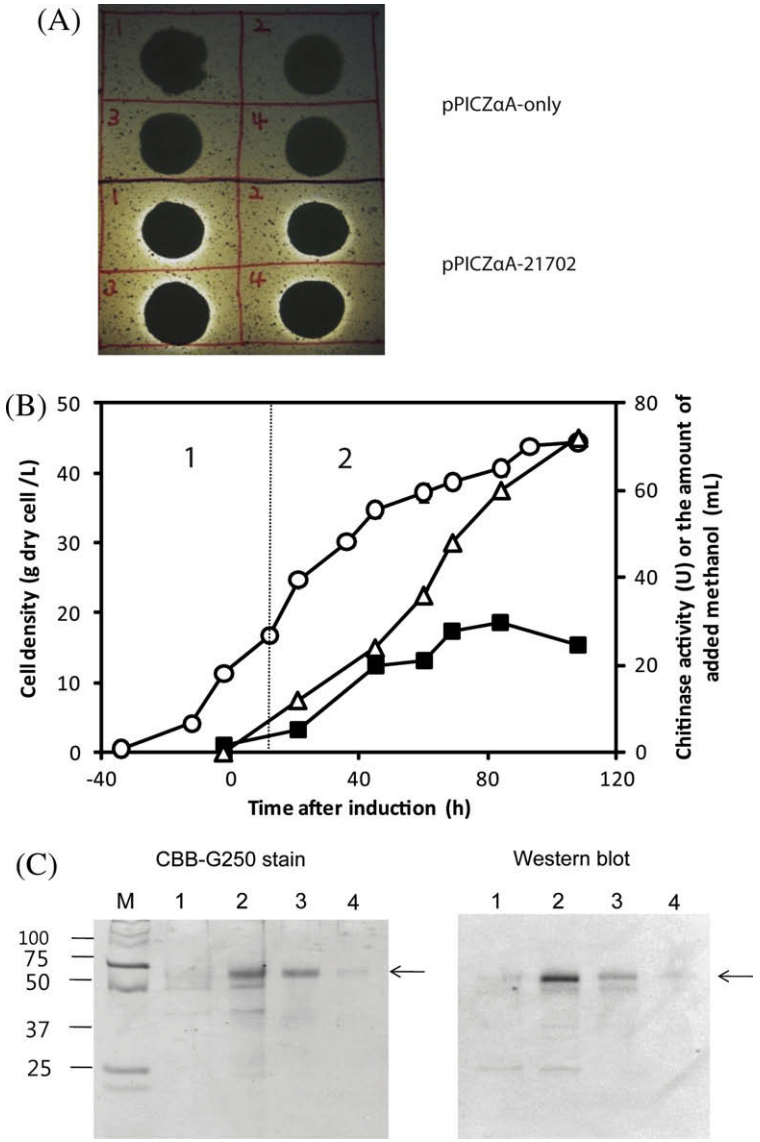


**Fig. 2.** Expression of rChi21702 in yeast. (A) Screening of rChi21702-secreting recombinant *Pichia* cells. Control cells transformed with pPICZαA vector did not show a clear zone around colony boundaries (pPICZαA-only). Cells harboring pPICZαA-chi21702 revealed a distinct clear zone around the colony boundaries (pPICZαA-21702). (B) Fermentation to produce rChi21702. Time courses of cell density (○), chitinase activity (■), and the amount of methanol feed medium added to fermenter (△) in batch (1) and fed-batch (2) culture. Dotted line indicates the start of medium supplementation with methanol. (C) Purification of rChi21702 expressed in *Pichia*. Elution buffer containing 1.5% (lane 1), 2% isopropanol (lane 2), 2.5% isopropanol (lane 3), and 3% isopropanol (lane 4) was applied to the Phenyl-Sepharose column sequentially to partially purify the expressed protein. The purified enzyme is indicated by an arrow.

*Purification of rChi21702*

After fermentation, two methods of affinity chromatography were applied to purify the rChi21702:cMyc and 6XHis tag columns. However, the enzyme did not bind to either affinity resin (data not shown). Since it was thought that the C-terminal tags might not be exposed to the water accessible site in the three-dimensional structure, a Phenyl-Sepharose column was used to purify the recombinant chitinase after 80% ammonium sulfate precipitation and dialysis against 20 mM Tris–Cl, pH 8.0. Eluates containing 2% isopropanol and 2.5% isopropanol showed >60% and >90% purities, respectively, as determined by scanning densitometry of CBB-G250-stained gels (Fig. 2C, lanes 2 and 3, respectively). The purified enzyme produced an immunoreactive signal by Western blot analysis using 6XHis antibody (Fig. 2C, Western blot). Both 2% and 2.5% eluates were combined and used for further enzymatic characterization. Ultimately, 1 mg rChi21702 was obtained from 1 L bioreactor culture (Table 1).

*Characterization of the purified rChi21702*

Specific enzyme activity of the purified rChi21702 was determined to be 20 U/mg using *p*-NP-$(GlcNAc)_2$ as the substrate, which is similar to that of native enzyme [22]. The optimal temperature for the activity of rChi21702 was 37 °C (Fig. 3, left), and >80% optimal activity was revealed at 20 °C, which demonstrates that the enzyme is suitable for the digestion of chitin at room temperature. Interestingly, the enzyme showed up to 63% activity at 10 °C and retained 44% activity at 0 °C (Fig. 3, left). The recombinant Chi21702 exhibited slightly different sensitivity to temperature when compared to that of wild-type Chi21702 (Fig. 3, left). The optimal pH for rChi21702 was found to be 7.6, which is similar to that of wild-type Chi21702 (Fig. 3, right). The enzyme retained almost no activity below a pH of 5.0. This indicates that the Chi21702 endochitinase is an acid-sensitive enzyme. Chitooligomers [$(GlcNAc)_{1-6}$] were digested with rChi21702, and the resulting products were analyzed by TLC (Fig. 4A). rChi21702 did not hydrolyze $(GlcNAc)_2$, but $(GlcNAc)_3$ was cleaved to produce GlcNAc and $(GlcNAc)_2$ after a 6 h incubation. The enzyme digested $(GlcNAc)_4$ and generated $(GlcNAc)_2$ as a major product with a small amount of GlcNAc. The major product of $(GlcNAc)_5$ and $(GlcNAc)_6$ digestion was $(GlcNAc)_2$, but a small amount of GlcNAc was detected after 6 h (Fig. 4A). When colloidal chitin was used as a substrate, $(GlcNAc)_2$ was clearly liberated within 1 h and its production increased over the next 4 h. Moreover, a small amount of GlcNAc was observed after 3 h, and was clearly present by 5 h (Fig. 4B). Considering all TLC data together, $(GlcNAc)_2$ was the major product resulting from catalysis by rChi21702, indicating that the enzyme is an endochitinase.

## Discussion

The methylotrophic yeast, *P. pastoris*, was used to express a recombinant Antarctic chitinase (rChi21702) because of its ability to generate large quantities of protein that is properly folded and easy to isolate [16,23]. The recombinant enzyme possessed endochitinase activity, as demonstrated using colloidal chitin, chitooligomers, and *p*-NP-$(GlcNAc)_2$ as substrates. Yeast (X33) transformed with the native chi21702 sequence yielded a recombinant enzyme with chitinolytic activity using *p*-NP-$(GlcNAc)_2$ as a substrate, but expression was poor, so that it was hardly able to detect the immunoreactive signal by Western blot analysis or enzyme activity (data not shown). To improve expression, differences in relative codon frequency between *S. antarcticus* and *P. pastoris* were considered, and the nucleotide sequence for rChi21702 was modified to match the codon bias for *P. pastoris* (Fig. 1A).

Chi21702 is a cold-adapted chitinase. Recombinant Chi21702 retained almost half of its optimal catalytic activity even at temperatures as low as 0 °C, and exhibited more than 60% of optimal activity in the temperature range from 10 to 37 °C (Fig. 3, left). Thus, the enzyme is suitable to produce GlcNAc at or below room temperature. Moreover, the psychrophilic feature of rChi21702

**Table 1**
Purification summary for rChi21702.

| Step | Volume (ml) | Protein (mg/ml) | Activity (U/ml) | Total activity (U) | Specific activity (U/mg) | Yield (%) | Fold purified |
|---|---|---|---|---|---|---|---|
| Culture medium | 1000 | 0.34 | 0.61 | 610 | 1.8 | 100 | 1 |
| Ammonium sulfate precipitation | 20 | 3.8 | 16 | 320 | 4.2 | 52.5 | 2.3 |
| Phenyl-Sepharose | 1 | 1 | 20 | 20 | 20 | 3.3 | 11.1 |

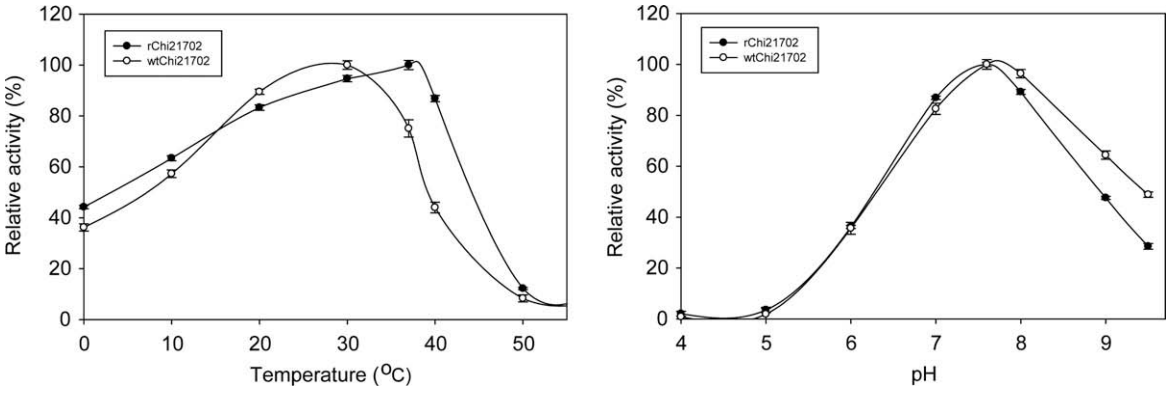


**Fig. 3.** Effect of temperature and pH on the activity of wild-type Chi21702 (wtChi21702) and recombinant Chi21702 (rChi21702). Enzyme activity at various temperatures (0–55 °C) was measured (solid circle, rChi21702; open circle, wtChi21702). Enzyme activity at various pHs (50 mM sodium acetate for pH 4–6 and 50 mM potassium phosphate for pH 7.0–9.5) was measured at 37 °C (solid circle, rChi21702; open circle, wtChi21702). All the experiments represent means of triplicate measurements.

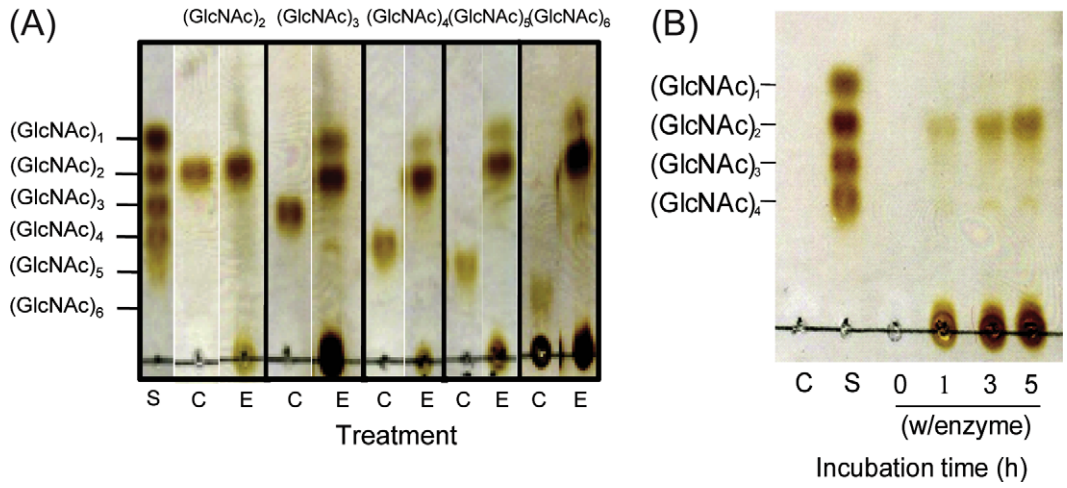


**Fig. 4.** TLC of digestion products by rChi21702. (A) Digestion products from various *N*-acetyl-chitooligosaccharides. The reaction mixtures (100 μl) containing each substrate in 50 mM sodium phosphate, pH7.6 were incubated with 50 ng rChi21702 for 6 h at 30 °C. Lane S, standard (G1:G2:G3:G4:G5:G6 = 4:2:2:2:1:1 mM); C, control reaction without enzyme; E, treatment with enzyme. (B) Digestion products from colloidal chitin demonstrated $(GlcNAc)_2$ generation. Lane C, negative control without enzyme for 5 h; lane S, standard (G1:G2:G3:G4 = 4:2:2:2 mM). Amount of $(GlcNAc)_2$ production was increased in a time-dependent manner. Each 10-μl aliquot was separated on a TLC plate with 2-propanol:water:ammonia solvent system, followed by developing using aniline-phthalate solution.

could be applied to non-antibiotic process in industry since any contaminating microbes can hardly grow at ice-cold temperature. It was reported that a psychrotolerant chitinase, ChiA, was expressed in *E. coli* and showed 40% of optimal activity at 5 °C [24]. When comparing rChi21702 and ChiA, we conclude that rChi21702 adapted to more extreme cold environment. Further biochemical and physiological studies are still remained to be elucidated in order to clarify the active mechanism of rChi21702 at ice-cold temperature.

As is the case for psychrotolerant endochitinases, very few psychrotolerant exochitinases have been discovered. In fact, very little GlcNAc produced by the industrial sector involves endochitinase digestion. Even though endochitinases liberate GlcNAc, the efficiency of the sole rChi21702 alone for the GlcNAc monomer is not sufficient enough because nonspecific cleavage activity of endochitinases generates mostly *N*-acetyl chitobioses. The production of GlcNAc would be accelerated by combining *N*-acetylglucosaminidases with endochitinases. Unfortunately, however, neither *N*-acetylglucosaminidase gene nor *N*-acetylglucosaminidase activity has been identified in the same strain of *S. antarcticus*. The other consideration would be the productivity of the enzyme. Unlike plant family 19 chitinases, the chitin binding domain of chi21702 gene was classified into family 18 chitinases by sequence homologies, which are found in bacteria, fungi, yeast, viruses, plant, and animals [25–27]. The codon usage of chi21702 was considerably biased in yeast cells, so the relatively low frequent bacterial codons were fully rearranged and the entire gene sequence was synthesized based on the result of optimization procedure (Fig. 1B). However, the yield of rChi21702 was 1 mg from 1-L bioreactor culture, even after codon optimization. It is still unclear why the expression level remained poor. In other reports, the expression of recombinant chitinases in *Pichia* system has been reported in milligram-to-gram level [28,29]. Endochitinase Ech42 from *Trichoderma atroviride* (61 mg) [29] and class III chitinase from *Oryza sativa* (1.25 g) [28] were produced to high yield from 1-L culture using *Pichia* expression system. Previous studies reported successful expression of enzymatically active recombinant endochitinases in *E. coli* [30,31]. An endochitinase A from *Vibrio carchariae* (100 mg) [31] and Ech42 from *Trichoderma harzianum* (1–2 g) [30] were obtained from 1-L culture of *E. coli* expression system. High-level expression systems of recombinant protein using *E. coli* to produce rChi21702 were attempted as well. The protein expression level was great, but all the protein resulted in the formation of insoluble inclusion bodies. Attempts to restore activity by refolding the enzyme were unsuccessful. Nonetheless, the present study addresses two basic questions on heterologous expression of an enzyme: expression of functional protein and improving the expression level. In conclusion, an Antarctic endochitinase rChi21702 was successfully expressed in *Pichia* and showed psychrophilic enzyme features. Considering that rChi21702 was produced in yeast cells, it is suitable for human or animal use.

## Acknowledgments

The authors wish to thank Dr. Hak Jun Kim and Ha Ju Park (Korea Polar Research Institute) for inspiring suggestions and Dr. Joseph A. Covi (Colorado State University) for his great efforts in improving the manuscript. This research was supported by a grant from the Korea Polar Research Institute (PE09050).

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