

Two-step purification of *Bacillus circulans* chitinase A1 expressed in *Escherichia coli* periplasm

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Abstract

A protein purification procedure was developed to efficiently and effectively purify the target enzyme, chitinase A1 of *Bacillus circulans* WL-12, from *Escherichia coli* DH5 α carrying the *chiA* gene with its natural promoter in the plasmid pNTU110. Chitinase A1 was purified to apparent homogeneity from *E. coli* periplasm with a final recovery of 90.6%. Two main steps were included in this protein purification procedure, ammonium sulfate precipitation (40% saturation) and anion-exchange chromatography at pH 6.0 using Q Ceramic HyperD column. The yield of chitinase A1 was estimated at 95 μ g/L. A polyclonal antibody against chitinase A1 was raised by immunizing BALB/c mice with chitinase A1 purified from *E. coli* DH5 α (pNTU110). As indicated by Western blot analysis, a 3000-fold diluted antibody detected purified chitinase A1 from *E. coli* DH5 α (pNTU110) in an amount of at least 1 ng and specifically detected chitinase A1 produced by *B. circulans* WL-12.

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Chitin, a β -1,4-linked homopolymer of *N*-acetylglucosamine (GlcNAc), is one of the most abundant polysaccharides found in nature [1]. It is degraded to GlcNAc monomers by the cooperative interaction of two enzymes, chitinase (EC 3.2.1.14) and β -*N*-acetylglucosaminidase (EC 3.2.1.30; GlcNAcase) [2]. Chitinases are found in a wide range of organisms, including bacteria, fungi, higher plants, insects, crustaceans, and some vertebrates [1–4]. They play important physiological and ecological roles in chitin metabolism of microorganisms for the production of carbon and energy sources and the antagonism toward target fungi [1–3]. In higher plants which do not contain chitin constituent, production of chitinase is generally involved in the defense mechanisms against fungal pathogens [1,2].

Bacillus circulans WL-12, a Gram-positive bacterium, secretes chitinases that are encoded by three genes (*chiA*, *chiC*, and *chiD*) [5–8]. Chitinase A1, encoded by *chiA*, is a key enzyme in the chitinolytic system of *B. circulans*

WL-12 [9]. It has been studied from several aspects including gene structure [7] and catalytic mechanism [10–14]. In this study, we proposed a two-step procedure to purify chitinase A1 from *Escherichia coli* cells harboring *chiA* gene based on the trait of low isoelectric point (pI) of this protein [7,9]. This purified chitinase A1 was used in raising polyclonal antibody specific for the detection of corresponding chitinase produced by *B. circulans* WL-12 and could be useful for the study of possible function of chitinase A1 toward fungi.

Materials and methods

Bacterial strains and growth conditions

Escherichia coli DH5 α was used as an *E. coli* host for transformation and protein expression. Luria–Bertani (LB) medium (1% Bacto-tryptone, 0.5% Bacto-yeast extract, and 0.5% NaCl, pH 7.0; 2% agar was added for solid medium) was used for general purposes and supplemented with 50 μ g/ml ampicillin if required. *B. circulans*

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WL-12 [15], a bacterial stock from Culture Collections and Research Center in Taiwan (originally from German Collection of Microorganisms and Cell Cultures, DSM596), was used as the source of *chiA* gene and cultured in LB medium supplemented with 0.2% colloidal chitin. Colloidal chitin was prepared from crab shell chitin (Sigma). Ten grams of crab shell chitin was mixed with 300 ml of 85% phosphoric acid and stirred at 4°C for two days. The resulting hydrolysate was neutralized by washing with distilled water and collected as colloidal chitin after centrifugation at 1500g for 10 min. All bacteria were cultured at 37°C.

Construction of recombinant plasmid for the expression of chitinase A1

Genomic DNA of *B. circulans* WL-12 was isolated according to a procedure described elsewhere [16] and used as the template for amplification of *chiA*-containing fragment with oligonucleotide primers, 5'-AGC GGCTGGAGGCGGCTATACGGC-3' and 5'-CTAA ACTAAGCTCGCCAACACTGC-3', by polymerase chain reaction (PCR). PCR thermal profile consisted of 30 cycles of 95°C for 1 min, 50°C for 2 min, and 72°C for 2 min, followed by a final extension step at 72°C for 10 min. The amplified DNA fragment of 2.4 kb was cloned into pCR2.1-TOPO (Invitrogen). The sequence of insert DNA was determined by an ABI-310 autosequencer (Applied Biosystems) and compared with the sequence in GenBank Accession No. M57601. Production of chitinase of *E. coli* DH5 α cells carrying recombinant plasmid was confirmed by the enzyme assay using proteins from the periplasmic fraction of transformants [17].

Preparation of crude enzyme extract from E. coli cells for purification of chitinase A1

A 3 ml overnight culture of *E. coli* DH5 α carrying the recombinant plasmid was added to 200 ml fresh LB medium supplemented with ampicillin and cultured for 20–24 h to express chitinase. Crude enzyme extract was prepared from *E. coli* periplasm by an osmotic shock method [17]. Bacterial cells were harvested by centrifugation at 8000g at 4°C for 10 min and suspended in 10 ml cold (4°C) spheroplast buffer [100 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 0.5 mM sucrose, and 20 μ g/ml phenylmethylsulfonyl fluoride (PMSF)]. After incubation for 5 min on ice, bacterial cells were collected by centrifugation at 8000g at 4°C for 10 min and re-suspended in 6.67 ml cold sterile water supplemented with a proteinase inhibitor cocktail (Roche). This bacterial suspension was incubated for 45 s on ice and subsequently mixed with 334 μ l of 20 mM MgCl₂ (a final concentration of 1 mM). The supernatant of nearly 7 ml was collected by centrifugation at 8000g at 4°C for 10 min as the periplasmic fraction.

Purification of chitinase A1

Twenty milliliters of pooled periplasmic fractions was prepared from 600 ml culture of *E. coli* DH5 α carrying the recombinant plasmid. All subsequent purification steps were performed at 4°C. Proteins in the periplasmic fractions were fractionated by adding solid ammonium sulfate to 40% saturation. The precipitate was dissolved in 1 ml of 25 mM potassium phosphate buffer, pH 6.0, containing 1 mM PMSF and dialyzed overnight against the same buffer. The dialysate was applied to a 5 ml anion-exchange (Q Ceramic HyperD) column (Sigma) equilibrated with the same buffer. Proteins were eluted with NaCl of 0.1, 0.3, and 0.5 M in 25 mM potassium phosphate buffer, pH 6.0. Aliquot fractions (1 ml each) were collected. Proteins obtained from each step were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and in-gel chitinase activity assay.

Protein concentration determination

Protein concentration was determined by the method described by Bradford [18] using a protein assay dye reagent concentrate (Bio-Rad). Bovine serum albumin was used as standard.

Enzyme assay

The enzymatic activity by using assay with fluorogenic 4-methylumbelliferyl-*N,N,N'*-triacetylchitotriose (4-MU-(GlcNAc)₃) [10] was measured by a fluorescence spectrophotometer (F-4500, Hitachi). One unit of chitinase activity was defined as the amount of enzyme required to release 1 μ mol of 4-methylumbelliferone (4-MU) per minute.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

SDS–PAGE in 10% separating gel containing 0.01% glycol chitin was carried out as described by Ames [19] using the buffer system of Laemmli [20]. Duplicate gels were run. After electrophoresis, one gel was soaked in 0.1 M sodium acetate buffer (pH 5.0) containing 1% Triton X-100 for protein renaturation, then the gel was stained with 0.01% Calcofluor White M2R in 0.5 M Tris-HCl (pH 8.9). Protein bands exhibiting chitinase activity were visualized under UV transilluminator [21]. In addition, proteins in another gel were stained with Coomassie brilliant blue G-250.

Preparation of antibody

Polyclonal antibody against purified chitinase A1 was raised in 4-week-old BALB/c mice. Chitinase of 100 μ g was applied as immunogen in the initial immunization of

BALB/c mice and succeeding injections 2 and 4 weeks thereafter.

Preparation of crude chitinases from the culture supernatant of *B. circulans* WL-12

Three-day culture of *B. circulans* WL-12 was centrifuged at 10,000g at 4°C for 15 min. Proteins in the culture supernatant were precipitated with 10% trichloroacetic acid at 4°C for 30 min. The precipitated proteins were subsequently collected by centrifugation at 10,000g at 4°C for 15 min and suspended in 0.1 M Tris–HCl buffer (pH 8.0).

Western blot analysis

Western blot analysis was performed to analyze polyclonal antibody against chitinases from *E. coli* cells expressing the *chiA* gene and the culture supernatant of *B. circulans* WL-12. Immunoreactive proteins were detected by an enzyme immunoassay using peroxidase-conjugated goat anti-mouse immunoglobulin (Amersham Biosciences) as secondary antibody and diaminobenzidine as a substrate of peroxidase.

Results

Purification of chitinase A1 from *E. coli* DH5 α harboring pNTU110

The PCR-amplified DNA containing *chiA* gene and its promoter region was ligated into pCR2.1-TOPO to generate plasmid pNTU110. The *E. coli* DH5 α cells carrying pNTU110, exhibiting chitinase activity, were used for the production of chitinase A1. Chitinase A1 in the periplasmic fraction of *E. coli* DH5 α (pNTU110) was purified to apparent homogeneity by a two-step procedure that included ammonium sulfate precipitation and anion-exchange chromatography. Approximately 33.7-fold purification was achieved with 90.6% yield. Overall yield of purified chitinase A1 was estimated at 95 μ g/L. The specific activity of purified chitinase A1 was determined to be 2.193 U/mg. The degree of purification and yield at individual steps are given in Table 1. As shown in Fig. 1, chitinase was mostly eluted with NaCl at a

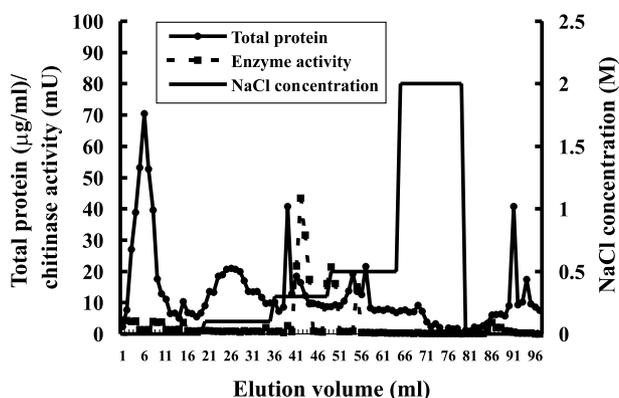


Fig. 1. Profile of chitinase A1 purification by anion-exchange chromatography. The proteins were eluted stepwise with NaCl of 0.1, 0.3, 0.5, and 2 M in 25 mM potassium phosphate buffer, pH 6.0. Most of the target protein, chitinase A1, was eluted by 0.3 M NaCl.

concentration of 0.3 M in 25 mM potassium phosphate buffer, pH 6.0, in anion-exchange chromatography, as indicated by the assay of chitinase activity. Smaller amounts of chitinase bound to anion exchanger were eluted with NaCl at a concentration of 0.5 M in 25 mM potassium phosphate buffer, pH 6.0. In addition, at least two peaks without chitinase activity appeared before elution of chitinase protein, which were proteins of higher *pI* values than that of chitinase A1 (*pI* 4.7).

Electrophoresis and in-gel chitinase activity assay

In SDS–PAGE and in-gel chitinase activity assay, two major protein bands appeared in ammonium sulfate-precipitated periplasmic protein sample of *E. coli* DH5 α harboring pNTU110 (Fig. 2A). One of the bands corresponded to chitinase A1 as shown by in-gel activity assay. Smaller proteins exhibiting chitinase activity appeared in the gel, similar to that shown in the sample of periplasmic fraction (Fig. 2B). However, the other major band did not exhibit chitinase activity. After anion-exchange chromatography, only a major protein band was visualized in the protein samples eluted with 0.3 M NaCl in 25 mM potassium phosphate buffer, pH 6.0, which showed chitinase activity as indicated by fluorometric assay (Fig. 2A). This purified protein exhibited chitinase activity in the in-gel activity assay and smaller proteins with chitinase activity became scarce (Fig. 2B).

Table 1
Purification of chitinase A1 from *E. coli* DH5 α harboring pNTU110

Purification step	Total activity (U) ^a	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yield (%)
Periplasmic fraction	0.138	2.130	0.065	1	100
Ammonium sulfate precipitation	0.109	0.145	0.752	11.6	79.0
Anion-exchange chromatography	0.125	0.057	2.193	33.7	90.6

^a 1 U of activity = 1 μ mol of 4-MU released per minute.

Specificity assay of polyclonal antibody against chitinase A1

The polyclonal antibody against chitinase A1, prepared in BALB/c mice, was examined for its sensitivity and specificity by Western blot analysis. A 3000-fold diluted antibody detected purified chitinase A1 from *E. coli* DH5 α (pNTU110) at an amount of at least 1 ng and specifically detected chitinase A1 produced by *B. circulans* WL-12 (Fig. 3). A degradation product of chitinase A1, present in the sample of periplasmic fraction of *E. coli* DH5 α (pNTU110), was detected by polyclonal antibody against chitinase A1. However, this degradation product of chitinase A1 did not hydrolyze glycol chitin and exhibited chitinase activity in the in-gel activity assay (Fig. 3).

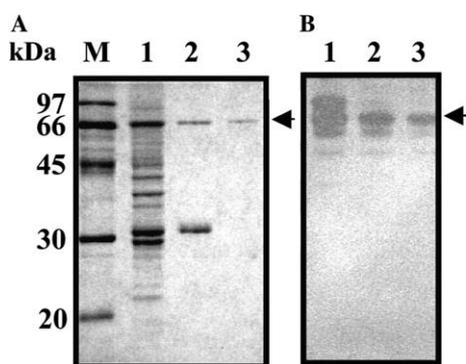


Fig. 2. SDS-PAGE analysis in each step of chitinase A1 purification. Periplasmic fraction of *E. coli* DH5 α (pNTU110) (lane 1), partially purified proteins from ammonium sulfate precipitation and overnight dialysis with 25 mM potassium phosphate buffer, pH 6.0 (lane 2), and the chitinase-containing fraction eluted with 0.3 M NaCl in 25 mM potassium phosphate buffer, pH 6.0, by anion-exchange chromatography (lane 3) were analyzed. Proteins in the polyacrylamide gels containing 0.01% glycol chitin were stained with Coomassie brilliant blue G-250 (A) and the presence of chitinase was detected by in-gel activity assay (B). The bands corresponding to chitinase A1 are indicated by arrows. M, low molecular weight standards (Amersham Biosciences).

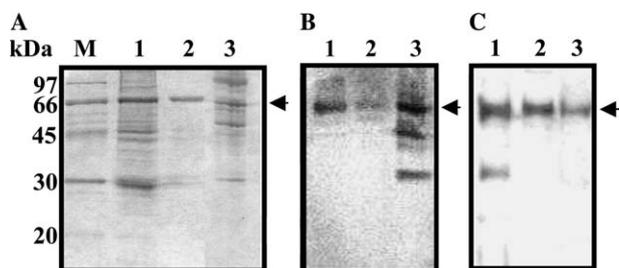


Fig. 3. Specificity assay of polyclonal antibody against chitinase A1. Periplasmic fraction of *E. coli* DH5 α (pNTU110) (lane 1), chitinase A1 purified by anion-exchange chromatography (lane 2), and the proteins in 3-day culture supernatant of *B. circulans* WL-12 (lane 3) were analyzed by SDS-PAGE in polyacrylamide gel containing 0.01% glycol chitin. The gels were stained with Coomassie brilliant blue G-250 (A) or subjected to in-gel activity assay (B). Western blot analysis showed specificity of the polyclonal antibody against chitinase A1 produced by *B. circulans* WL-12 (C). The bands corresponding to chitinase A1 are indicated by arrows. M, low molecular weight standards.

Discussion

Chitinase A1 has been identified as a key enzyme in hydrolyzing chitin by *B. circulans* WL-12 and its encoding gene, *chiA*, was cloned and sequenced [7,9]. The *chiA*-transformed *Bacillus subtilis* F29-3 expressed higher antifungal activity than wild-type bacterium against *Botrytis elliptica*, a fungal pathogen of lily leaf and flower blight [22]. Presumably, chitinase encoded by the *chiA* gene may increase the function of antifungal metabolites produced by *B. subtilis* F29-3.

To study the possible function of chitinase A1 toward fungi, pQE vectors (Qiagen) were first used for the expression of chitinase A1 in *E. coli*. However, this approach was not a success in our study. Therefore, *E. coli* cells harboring plasmid pNTU110 were used to moderately express chitinase A1 for protein purification. For the purification of chitinase A1 from *E. coli* cells harboring pNTU110, chitin affinity chromatography was first followed [7,13], but the yield of chitinase after chitin adsorption step in our procedure was very low. The low efficiency was possibly due to the difficulty in manipulating chitin affinity chromatography or the quality of the regenerated chitin. To prepare a sufficient amount of protein for our study on chitinase A1, an easy and rapid purification procedure was therefore developed.

The major viewpoint of protein purification procedure developed in this study is based on the fact that chitinase A1 has a low *pI*, thus making it negatively charged at pH 6.0. After ammonium sulfate precipitation, the protein pellet was dissolved in potassium phosphate buffer, pH 6.0. In this condition, the negative charge of most *E. coli* proteins [23,24] would be much weaker than the chitinase charge. Since the strong anion exchanger, Q Ceramic HyperD ion exchanger, could maintain a high ability of anion exchange at pH 6.0 to absorb negatively charged proteins such as chitinase A1, this protein could be subsequently eluted by NaCl-containing buffer at certain concentration. The result verified that our proposed procedure could purify chitinase A1 from *E. coli* periplasm to near homogeneity. The flow-through fraction in anion-exchange chromatography should include *E. coli* proteins positively charged at pH 6.0 [23,24]. The procedure to purify *B. circulans* chitinase A1 from *E. coli* harboring *chiA* gene using ion exchange at acidic pH is our own invention. Purified chitinase A1 was employed to prepare polyclonal antibody which could specifically detect chitinase A1 without cross reactivity to other unrelated chitinases produced by *B. circulans* WL-12 [7,9] and might be useful as an immunological tool in further studies related to chitinase A1.

The concept to develop an effective and efficient procedure of chitinase A1 purification in this study is based on the low *pI* of the target protein and the characteristic of the strong ion exchanger, which was first applied to

purify *B. circulans* chitinases. A similar strategy may be employed to purify proteins with low pI value as chitinase A1.

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