J. Microbiol. Biotechnol. (2010), **20**(7), 1114–1120
doi: 10.4014/jmb.0909.09031
First published online 13 May 2010

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Isolation, Characterization, and Application of Chitosan-Degrading Fungus from Soil

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Received: September 20, 2009 / Revised: February 20, 2010 / Accepted: March 2, 2010

A chitosan-degrading fungus, BSF114, was isolated from soil. The culture preparation showed strong chitosanolytic enzyme activity at an optimum pH of 4.0 and optimum temperature of 60°C after 36–40 h fermentation. The rapid decrease in the viscosity of the chitosan solution early in the reaction suggested an endo-type cleavage of the polymeric chitosan chains. To identify the isolated fungus, molecular biological and morphological methods were used. The fungal internal transcribed spacer (ITS) region 1 was amplified, sequenced, and then compared with related sequences in the GenBank database using BLAST. The phylogenetic relationships were then analyzed, and the results showed that the fungus belongs to *Aspergillus fumigatus*. Morphological observations were also used to confirm the above conclusion. The chitooligosaccharides (COS) obtained through hydrolyzing the colloidal chitosan showed that *A. fumigatus* BSF114 is suitable for degrading chitosan and producing chitooligosaccharides on a large scale. High concentrations of the COS (1,000 and 500 μg/ml) significantly proliferated mice marrow cells.

Keywords: Chitooligosaccharides, identification, morphology, *Aspergillus fumigatus*

Chitosan and its derivatives exhibit various functional properties, making them useful in many fields, including food, cosmetics, biomedicine, agriculture, and environmental protection [2, 10]. Furthermore, the chitooligosaccharides (COS) from chitosan have a lower viscosity and greater solubility at a neutral pH than chitosan, which has attracted great interest from researchers with regards utilizing chitosan in its oligosaccharide form [10, 18]. For example, COS are used in food and nutrition to improve the quality of food and human health. Recent studies have also focused on the health benefits of COS, including their antimicrobial effect [22], abilities to lower blood cholesterol [5] and lower high blood pressure [6], and anticancer activity [8].

A wide range of chemical and enzymatic methods can be used for COS production, among which chemical hydrolysis is currently the most common for industrial-scale production [10]. Endo-type cleavage chitosanases (E.C. 3.2.1.132) are glycosyl hydrolases that catalyze the hydrolysis of the 1,4-glycosidic linkage of chitosan and thereby produce glucosamine oligosaccharides [1, 19, 24]. However, these enzymatic processes are generally carried out in batch reactors and are preferable over chemical methods [5, 10].

Thus, endo-type cleavage chitosanases play an important role in the large-scale production of COS [1, 19, 24]. Chitosanases have been reported to be widespread in microorganisms, including bacteria, actinomycetes, and fungi [11, 20]. Traditionally, morphological observations, and physiological and biochemical tests are used to identify a fungus. However, these methods are always time-consuming and laborious [17, 26]. Alternatively, an internal transcribed spacer (ITS) region is often used as the sequenced DNA region in fungi and has been considered as a useful tool for molecular systematics at the species level [6]. However, only a limited number of species and specific genera have been identified by ITS sequence analyses [13]. Moreover, the sole use of the ITS1 region for strain identification is relatively infrequent [15, 16]. Therefore, this study used both molecular biological methods and morphological observation to identify a chitosan-degrading strain from soil. A pair of universal primers, ITS1 and ITS2, was chosen to selectively amplify the ITS1 region. After sequencing these fragments, the phylogenetic relationships were then analyzed using bioinformatics [16, 21].

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MATERIALS AND METHODS

Screening of Bacterial Strains and Culture Conditions

The target strains were obtained from soil from a carapace recycling dump in Qi Dong, Jiangsu Province, China. The soil suspension was stirred with a magnetic stirrer and the diluted supernatant inoculated onto a medium plate consisting of 1% colloidal chitosan, 0.5% $(NH_4)_2SO_4$, 0.2% K_2HPO_4, 0.1% $MgSO_4$, and 2% agar (pH 6.5) at 32°C. Four days later, single colonies were observed and inoculated into 250-ml Erlenmeyer flasks with 100 ml of a liquid medium (the same ingredients mentioned above without the agar). Pure cultures were then obtained by successively re-inoculating the medium mixture into a chitosan agar.

Enzyme Assays

The fermented culture was collected after 36–40 h of shake-flask fermentation (liquid medium containing 1% chitosan powder, 0.5% peptone, 0.1% yeast extraction, 0.2% K_2HPO_4, and 0.1% $MgSO_4$) and the supernatant obtained by centrifugation was used as the enzyme source.

Chitosan with a degree of acetylation (D.A.) at 10% was used as the substrate in the standard chitosanase assay. Unless specially noted, chitosanase activities were detected using the dinitrosalicylic acid (DNS) method with D-glucosamine-HCl as the calibration standard [25]. One unit of enzyme activity was defined as the amount of enzyme catalyzing the production of 1 μmole of the reducing sugar per minute. The kinematic viscosity of the chitosan (1 g of chitosan was dissolved in 25 ml of a 0.2 M HAc-NaAc buffer and diluted to 100 ml with water) was measured at 40°C using an NDJ-5S electronic viscometer.

DNA Extraction

In this study, the genomic DNA from the lyophilized mycelium was extracted as previously described elsewhere [21]. The 2% CTAB extraction buffer was pre-heated at 65°C. Approximately 100 mg of the pulverized mycelium was then transferred to a 1.5-ml tube with 1 ml of CTAB and 20 μl of β-mercaptoethanol, and incubated at 65°C for 30–40 min. Next, 150 μl of potassium acetate (5 mol/l) was added to the tube, which was kept in ice for 30 min. The tube was then centrifuged at 9,180 ×*g* for 10 min, and the lysate extracted with an equal volume of phenol–chloroform–isoamyl alcohol (25:24:1). This procedure was repeated 3 to 5 times to remove all the protein. Thereafter, the supernatant was moved to another tube containing an equal volume of chloroform–isoamyl alcohol (24:1) and promptly centrifuged. To precipitate the DNA, a double volume of ethanol was added to the supernatant at −70°C for 30 to 50 min, and the DNA washed with 400 μl of alcohol (70%). Finally, the recovered DNA was dried and resuspended in 30 μl of ultrapure water (containing RNAse) and stored at −20°C.

DNA Sequencing

Pairs of oligonucleotide fungal universal primers were used for the amplification. Primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS2 (5'-GC TGCGTTCTTCATCGATGC-3') were designated to amplify the internal transcribed spacer 1 (ITS1) region and part of the 18S and 5.8S rRNA genes. The primers were synthesized using Invitrogen (Shanghai, China), and the PCR products stored at −4°C until used. The products were analyzed by electrophoresis on 1% (w/v) agarose gels stained with 8 μl (0.5 μg/ml) of an ethidium bromide solution, and the results visualized with UV illumination and photographed using a gel imaging system. Both strands of the PCR products were directly sequenced.

Sequence and Phylogenetic Analyses

The sequence obtained above was pre-aligned using the alignment and search tool BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) to collect a set of taxonomically representative fungal sequences. These sequences written in FASTA format were then edited for use with the CLUSTAL X (Version 1.83) multiple-sequence alignment program. Dendrograms were constructed using the maximum parsimony treeing algorithm in PHYLIP (Version 3.66). Other programs, including TREEVIEW (Version Win32) and MEGA3.1, were also used to draw and edit the graphical trees. The phylogenetic trees were prepared using the maximum parsimony method, where the evolutionary distances between organisms were indicated by the horizontal branch lengths, reflecting the number of nucleotide substitutions per site along the branches from a node to the endpoint. The percentages of bootstrap samplings supporting the interior branches were also noted.

Morphological Observation

A drop of a potassium hydroxide solution (20%) and small amount of hyphae and spores were added to a slide and covered with a glass slip. The microscopic analysis was then performed using an optical microscope. For scanning electron microscopy (SEM), the spores were directly adhered to double-sided adhesive tape fixed to an observation stub, which was metalized with gold.

Preparation of Chitooligosaccharides

The COS were prepared through the hydrolysis of colloidal chitosan by the enzyme source obtained from *A. fumigatus* BSF114. First, 1.0 ml of the enzyme solution (4.0 units/ml) was added to 9.0 ml of 3.0% (w/v) colloidal chitosan. The mixture was then incubated for 12 h at 50°C. The reaction was stopped by the addition of 1 ml of trichloroacetic acid (50%) and maintained at 4°C for 3 h. An NaOH solution was added to adjust the pH to 8.0. Thereafter, the mixture was centrifuged to remove the chitosan, and the supernatant containing the COS collected. SephadexG-25 chromatography was used to purify the crude COS, and TLC [ethyl acetate:ethanol: H_2O:ammonia=5:5:4:0.3 (v/v/v/v)] and HPLC were used to check the concentration of COS. The HPLC analysis was performed on a Shodex Asahipak NH2P-50 4E column (4.6 mm×250 mm) using a differential refractive index detector. The mobile phase consisted of acetonitrile:water [70:30 (v/v)] at a flow rate of 1.0 ml/min.

RESULTS AND DISCUSSION

Acquisition of Chitosan-Degrading Strains

Considering that strains living in a medium with chitosan as the sole carbon source are quite rare, all the visible colonies were transferred to flasks and screened. Finally, by comparing the chitosanase activities, a filamentous fungus (BSF114) was selected as the object for further study. COS have received much attention for their excellent biological activities, and many chitosan-degrading strains have already been identified. Thus, most recent studies

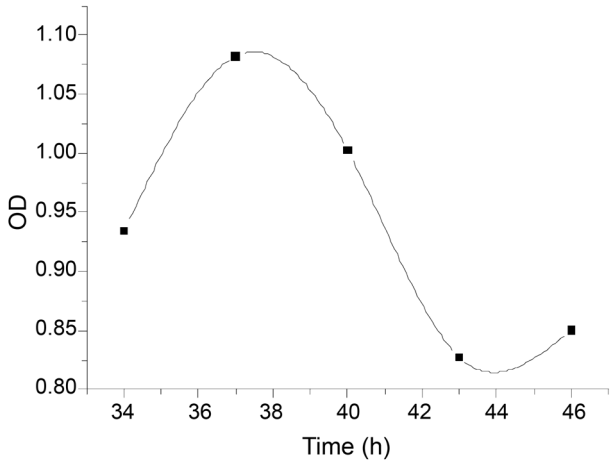

Fig. 1. Effect of time on enzyme activity.

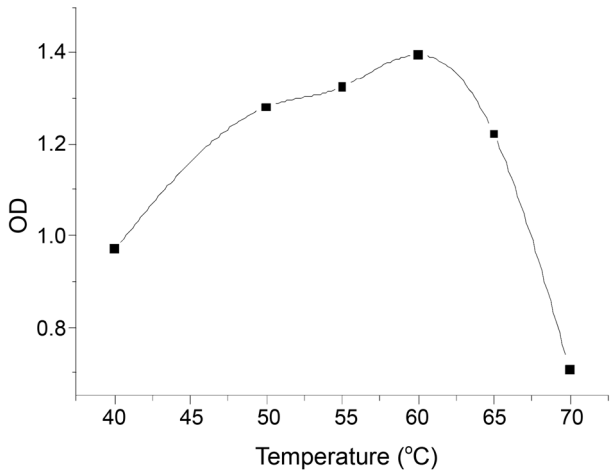

Fig. 3. Effect of temperature on enzyme activity.

have mainly been focused on the characteristics of chitosanases and qualities of COS [5].

Enzyme Analyses

Fig. 1 shows the time–activity curve for the fermentation of the enzyme produced from BSF114, which was maximized after fermentation in a flask for 36–40 h. Fig. 2 and 3 show that the optimum pH and temperature for preparing the enzyme were 4 and 60°C, respectively. Moreover, the kinematic viscosity test showed that the viscosity of the chitosan solution decreased over time when the reaction mixture was incubated for 1 h at 40°C (Fig. 4). The rapid decrease in the viscosity of the chitosan solution during the early stage of the reaction suggested an endo-type cleavage in the polymeric chitosan chains [9]. Thereafter, the functional characteristics of the enzyme were investigated. The enzyme showed an obvious hydrolyzing function on filter papers and cotton, yet had no effect on CMC-Na.

Sequence Analysis of ITS1 Region in rDNA Gene

To identify the isolated strain, the ITS1 region was selected as the specific sequence for amplification. The sole application of the ITS1 sequences in the rDNA is rarely used to identify a mold [19, 20]. However, in this study, pairs of universal primers (ITS1 and ITS2) were successfully used to amplify the target area. The amplifying range included partial sequences of the 18S rDNA and 5.8S rDNA and the whole ITS1 fragment. A DNA electrophoresis photograph of the internal transcribed spacer region (ITS1) is shown in Fig. 5A, which illustrates that the genomic DNA sequence was about 20,000 bp. Meanwhile, an electrophoresis analysis showed that the target DNA sequence was about 250 bp (Fig. 5B).

Phylogenetic Relationship and Dendrogram

When applying the resulting sequence to the GenBank DNA database, hundreds of related sequences were found.

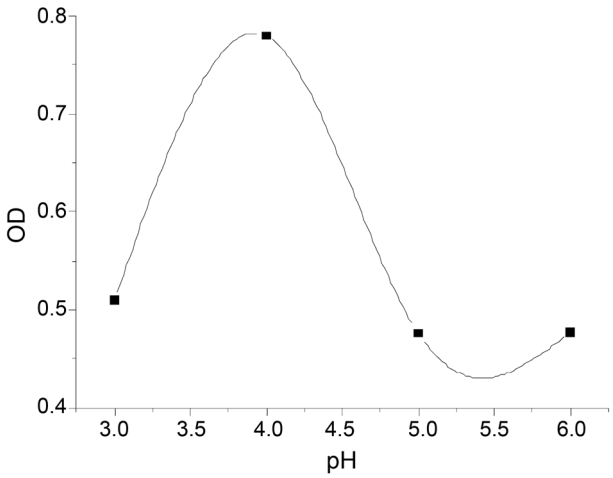

Fig. 2. Effect of pH on enzyme activity.

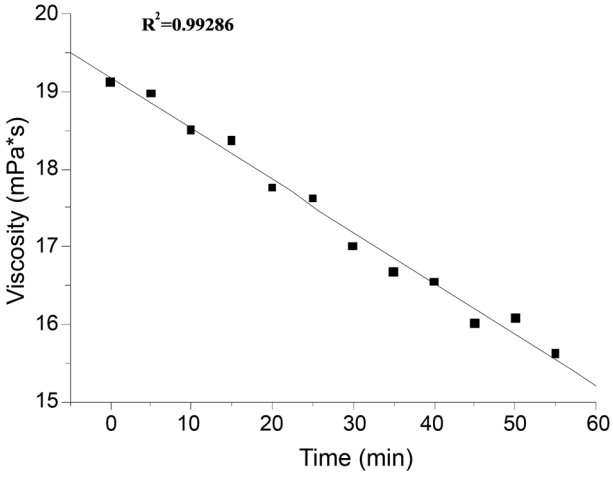

Fig. 4. Kinematic viscosity of chitosan at 40°C.

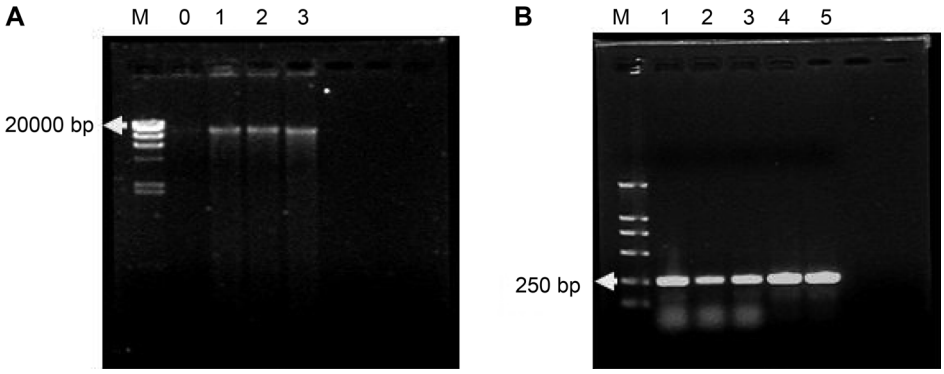

Fig. 5. DNA electrophoresis photographs of internal transcribed spacer region (ITS1) (**A**) and electrophoresis photograph of resultant PCR products (**B**).
M, marker; 0, blank.

Among these species, *A. fumigatus* represented an overwhelming majority. Several representative gene sequences were selected as phylogenetic relationship references, and phylogenetic trees were prepared using the maximum parsimony method. The evolutionary distances between the organisms were indicated by the horizontal branch length, reflecting the number of nucleotide substitutions per site along the branches from a node to the endpoint. The percentages of bootstrap samplings supporting the interior branches were also noted. First, different sources of organisms listed in BLAST were chosen to ascertain the possible evolutionary direction of BSF114 (data not shown). Tree A indicated that BSF114, AB051071, and AB197939 (Accession No.) belong to the same group, which consists of *A. fumigatus* (90% bootstrap support). Diverse sources of *A. fumigatus* were then selected to examine the evolutionary relations further. In tree B, BSF114, AB051071, and AB197939 were again grouped into one cluster (96% bootstrap support) (data not shown). In addition, the two trees showed that BSF114 had the closest phylogenetic relationship with strain AB051071. Thus, the molecular experiment and dendrogram results demonstrated that the target strain belonged to *A. fumigatus*.

Morphological Identification

BSF114 is a rapid-growth strain, and the color of its colonies is green or dark green. BSF114 showed stable growth activity when the culture temperature ranged from 28°C to 37°C, indicating that it is a thermophilic strain, which is the typical conidiophore of *Aspergillus*. The chains of the conidia are borne directly on broadly clavate vesicles (20 to 30 μm in diameter) and are hyaline and spheric (Fig. 6). Moreover, SEM micrography (Fig. 7) showed rough echinula structures (Fig. 7A and 7B) on the surface of the conidia, considered a typical characteristic of *A. fumigatus* [12]. Thus, all the morphological characteristics of BSF114 indicated that the strain belongs to *A. fumigatus*, demonstrating that the ITS1 region can be used to identify *A. fumigatus*. Morphological studies require related knowledge, and it is always difficult to orientate the desired points quickly. In contrast, molecular biological methods avoid such blindness, and can quickly narrow the

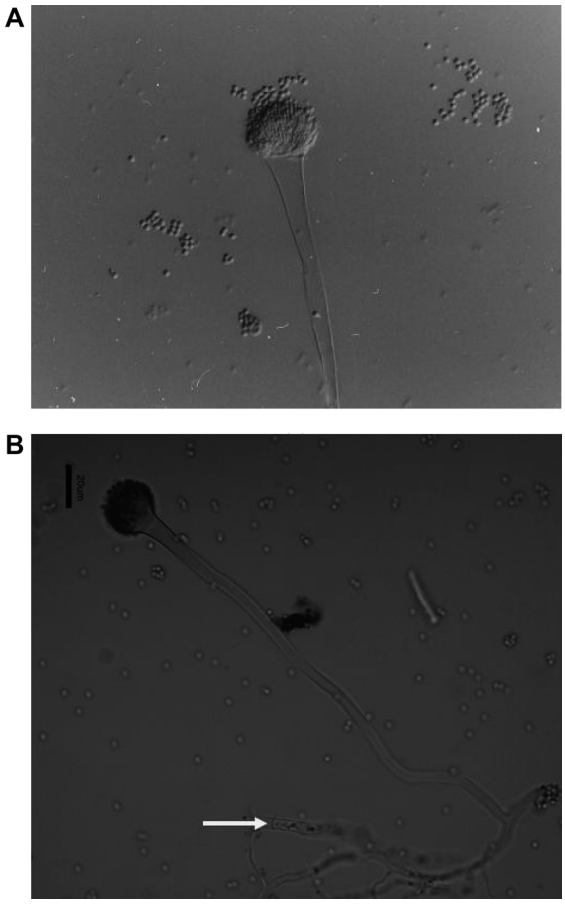

Fig. 6. Light microscopic photo of BSF114 in this study (**B**) compared with photo of typical *A. fumigatus* (**A**) referred by Latge [12].
The white arrow indicates the hypha diaphragm. The scale in B is equal to 20 μm.

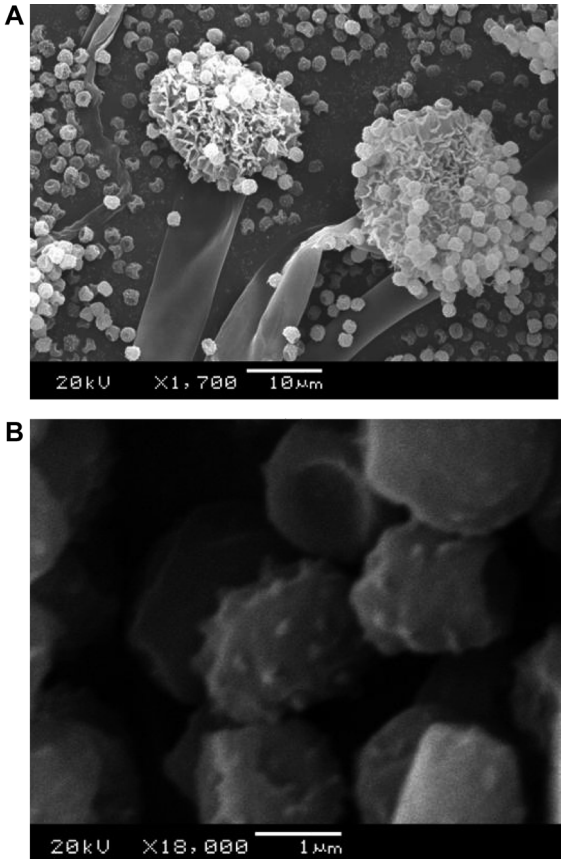

Fig. 7. Scanning electron microscopy (SEM) indicating conidiophores (**A**) and conidia (**B**) of BSF114.
A rough echinula structure can be seen clearly on the surface of the conidia. As the sample was not pretreated by procedures like fixing, dehydration, and drying, most of the spores shown in the photographs are sunken.

study scope. Thus, using the two methods in turn proved to be an effective way to identify a fungus.

In related studies, some *A. fumigatus* have been reported to have the ability to produce chitinases or chitosanases [4, 23]. Notwithstanding, the advantages of *A. fumigatus*, such as a quick growth property and strong ability to yield spores, which are propitious for industrial fermentation, need to be balanced with its disadvantages as a significant pathogen for immunocompromised hosts that causes pneumonia and invasive disseminated disease with a high mortality [5, 12]. Therefore, the biosafety aspects should be taken into account by further research.

Preparation of Chitooligosaccharides

COS were prepared through the hydrolysis of colloidal chitosan by the enzyme source obtained from *A. fumigatus* BSF114. The average molecular mass (m) of the resulting COS was found to be 1,389 Da, the viscosity [η] was 3.5 cP (5%), and the deactylation degree (DA) was 91%, as determined by titration. Fig. 8A shows the molecular

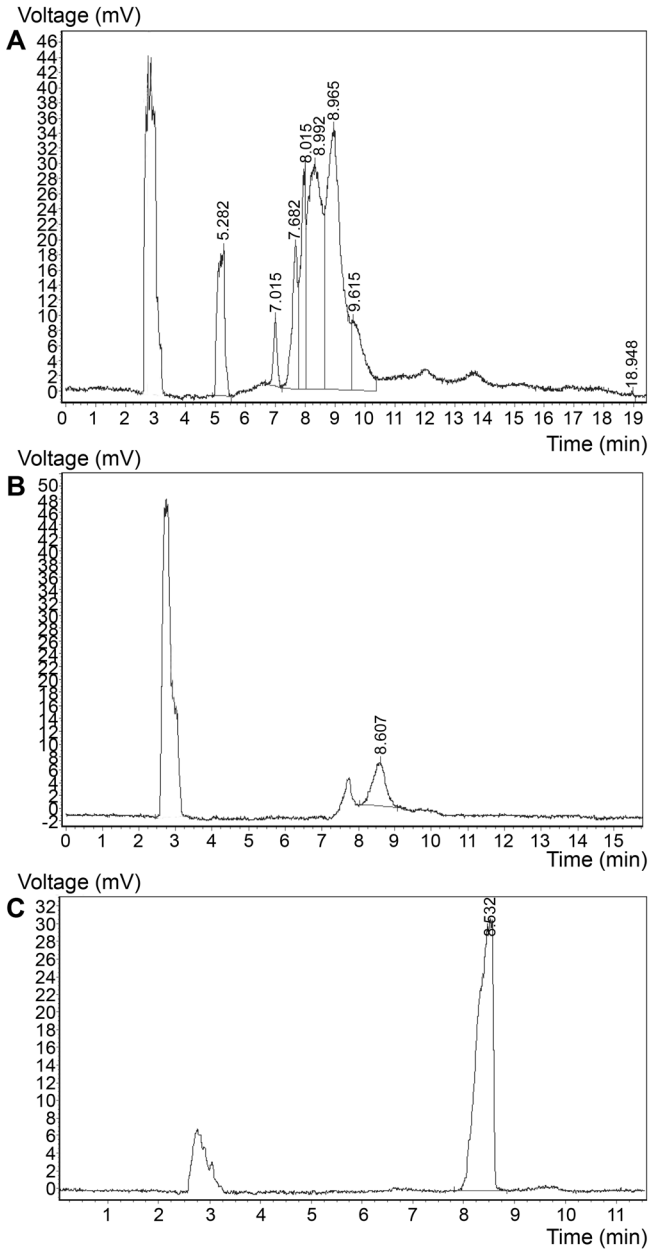

Fig. 8. HPLC profiles of enzyme reaction products.
A. Crude product from soluble chitosan incubated with crude enzyme; **B**. Purified product from Sephadex G-25 chromatography; **C**. Chitooligosaccharides obtained commercially.

weight distribution profile of the crude COS obtained by the enzyme hydrolysis, Fig. 8B shows the molecular weight distribution profile of the COS purified using Sephadex G-25 column chromatography, and Fig. 8C shows the molecular weight distribution profile of COS obtained commercially. When comparing Fig. 8A with Fig. 8B, it was found that Sephadex G-25 column chromatography is a suitable method for purifying crude COS. Moreover, when comparing Fig. 8B with Fig. 8C, it was concluded that the components of the COS obtained

Table 1. Proliferation effect of COS on mice marrow cells (*$P \leq 0.01$).

[COS] (μg/ml)	OD (570 nm)
0	0.4372±0.0066
1,000	0.5022±0.0071*
500	0.4609±0.0027*
50	0.4352±0.0125
5	0.4384±0.0030
0.5	0.4359±0.0050

by the enzyme hydrolysis were similar to those in the commercial products. Thus, *A. fumigatus* BSF114 would appear to be suitable for degrading chitosan and producing COS on a large scale. However, further work needs to focus on the parameters for industrial application.

Proliferation Effect of COS on Mice Marrow Cells

The effect of the COS obtained in this study on mice marrow cells was studied by measuring the cell numbers using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay after treatment with the COS for 72 h. A high concentration of COS (1,000 and 500 μg/ml) significantly proliferated the mice marrow cells (Table 1). However, a lower concentration of COS (0.5–50 μg/ml) did not have any evident proliferation effects on the marrow cells (Table 1). As shown in Table 1, the COS obtained in this study had no toxicity effect on the marrow cells.

Acknowledgments

The authors are grateful for the financial sponsorship from the National High Technology Research and Development (863) Program of China (2008AA10Z322), Shanghai Biomedicine Key Program (No. 08391911100), Shanghai Basic Research Key Program (No. 09JC1411500), and Shanghai Municipal Education Commission Program (No. 2005DZ28).

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