Cloning and Expression of Chitinase A from *Serratia marcescens* for Large-scale Preparation of *N,N*-Diacetyl Chitobiose

Yue-Jin Wu[a] (吳岳進), Chih-Yu Cheng[b]* (鄭至玉) and Yaw-Kuen Li[a]* (李耀坤)
[a]*Department of Applied Chemistry, National Chiao Tung University, Hsinchu, Taiwan, R.O.C.*
[b]*Department of Marine Biotechnology, National Kaohsiung Marine University, Kaohsiung, Taiwan, R.O.C.*

The gene of *Serratia marcescens* chitinase A (*chi*A) was cloned by PCR. The complete gene was constructed into a pRSET vector and expressed in *Escherichia coli*. The recombinant enzyme was purified to > 90% homogeneity by hydrophobic interaction chromatography followed by ion-exchange separation. Measured with an electrospray-ionization mass spectrometer, the molecular mass of the protein was 58,607 Da, consistent with a theoretical calculation of the deduced protein without the signal peptide. The recombinant enzyme was characterized and tested for the preparation of chitobiose. In general, the recombinant Chtinase A exhibited an exo-type catalytic activity toward colloidal chitin and released both *N*-acetylglucosamine and *N,N*-diacetyl chitobiose as products. After extensive testing, we produced *N,N*-diacetyl chitobiose as the predominant product when the enzymatic reaction was performed in sodium acetate buffer at pH 5.5; under such conditions, an enzymatic process is established for the production of the disaccharide on a 100-g scale.

Keywords: *Serratia marcescens*; Chitinase A; Colloidal chitin; *N*,*N*-Diacetyl chitobiose.

INTRODUCTION

Chitin, derived primarily from the exoskeletons of insects and crustaceans, is a β-1, 4-linked, insoluble linear polymer of *N*-acetylglucosamine (GlcNAc). It is the second most abundant organic compound on our planet next to cellulose and the major source of amino sugars in nature. The biological degradation of chitinous materials is thus an important process for the recycling of nutrients in the environment. Certain bacteria, plants and fungi secrete exo- or endo-chitinases capable of degrading chitin to its monomeric or oligomeric saccharides. Among them, exo-chitinase is most extensively studied including its protein structure, catalytic mechanism,[1] essential amino-acid residue(s),[2,3] and potential application for *N*,*N*-diacetyl chitobiose preparation.[4]

Although the biological function of chitooligosaccharide has not been well studied, the common feature of a pentasaccharide core containing a *N*,*N*-diacetyl chitobiose moiety and three mannose residues was found in many asparagine-linked glycoproteins, which dominates functions in vivo of protein folding,[5] receptor functioning, cell adhesion and signal transduction.[6] The biological functions of *N*,*N*-diacetyl chitobiose moiety in the glycan chain are thus expected to be significant. An example was found from the case of *E. coli* invasion in brain microvascular endothelial cells for which the recognition of *N*,*N*-diacetyl chitobiose epitopes on the glycoprotein of brain cell by *E. coli* is essential.[7,8] In addition to this physiological function, *N*,*N*-diacetyl chitobiose is an effective inducer of bacterial chitinase,[9] a pathogen-resistant enzyme against fungal infection.[10] We expect that further applications of *N*,*N*-diacetyl chitobiose would be developed if a source of this expensive disaccharide could be devised.

Methods developed to prepare *N*,*N*-diacetyl chitobiose include semi-biochemical synthesis, chemical degradation of chitin followed by oligosaccharide separation and enzymatic hydrolysis of chitin. A semi-biochemical process involving catalysis of chitinase with an 1,2-oxazoline derivative of *N*-acetylglucosamine as a glycosyl donor[11] has been developed, but suffers from high cost and poor

* Corresponding author. Tel: +886-3-573-1985; Fax: +886-3-572-3764; E-mail: ykl@faculty.nctu.edu.tw (Yaw-Kuen Li);
Tel: +886-7-361-7141 ext. 3817; Fax: +886-7-301-1171; E-mail: cycheng@mail.nkmu.edu.tw (Chih-Yu Cheng)
Abbreviations: ChiA, chitinase A; GlcNAc, *N*-acetyl-D-glucosamine; $(GlcNAc)_2$, *N*,*N*-diacetyl chitobiose.

yield. Although the chemical degradation of chitin is costless, the laborious separation can only allow a small-scale isolation of *N*,*N*-diacetyl chitobiose or other chito-oligosaccharides. Although many researchers have shown some drawbacks, such as product inhibition, protracted reaction[4] and the contamination of monomer, in an enzymatic process, an easily obtained chitinase with a high product specificity was expected to be useful for the preparation of *N*,*N*-diacetyl chitobiose. We report here that a *chi*A gene from *S. marcescens* was cloned, over-expressed and applied to preparation of *N*,*N*-diacetyl chitobiose on a 100-g scale.

EXPERIMENTAL SECTION

Material

Chemical and microbes. Buffers Sigma-Aldrich (St. Louis, MO, USA), HiTrap SP and Q columns and phenylsepharose resin (Amersham Bioscience, Uppsala, Sweden), chitin, (local supplier in Taiwan) and *S. marcescens* (ATCC 990, Food Industry Research and Development Institute, Hsinchu, Taiwan) were obtained from the indicated sources.

Cloning of chiA

The *chi*A gene was PCR-amplified from genomic DNA of *S. marcescens* using primers 5'-GGAATCACATATGCGCAAATTTAA-3', and 5'-GCAACCGATTATTGAACGCCGG-3' which were designed on the basis of the *S. marcescens chi*A gene published in the GenBank database (accession number AF085718). PCR amplification was performed with *Vent* DNA polymerase (NEB) with 25 cycles; each cycle involved 94 °C, 30 s for denaturation, 60 °C, 30 s for annealing, and 72 °C, 4 min for extension. The PCR fragment was first cloned into a cloning vector (QuanTox, Stratagen Co.) and sequenced. The correct gene was then inserted into *Nde* I/*EcoR* I sites of pRSET A and expressed in *Escherichia coli*.

Cultural conditions and purification of Chitinase A (ChiA)

E. coli BL21 (DE3) served as the host strain for protein expression. A single colony was inoculated into LB medium (5 mL) containing ampicillin (0.1 mg/mL) and cultured at 37 °C on a rotary shaker for 12 h. The overnight culture was then transferred into a conical flask (2 L) containing LB medium (1 L) with ampicillin (0.1 mg/mL) at 37 °C for 15 h. The culture broth was centrifuged at 4 °C for 10 min at 7000 xg. The cell pellet was resupended in sodium phosphate buffer (10 mL, 20 mM, pH 7.0) and then subjected to cell disruption with ultrasonication. After centrifugation to remove cell debris, the supernatant (~10 mL) was loaded onto a hydrophobic interaction column (2.4 × 20 cm, high-performance phenylsepharose), which was pre-equilibrated with sodium phosphate buffer (20 mM, pH 7.0) containing $(NH_4)_2SO_4$ (1 M). Elution at flow rate 2 mL/min with a linear gradient of $(NH_4)_2SO_4$ from 1000 to 0 mM was performed at a decreasing rate 16.67 mM/min. Fractions with chitinase activity were collected and loaded onto a series connection of a cation-exchange column (5 mL HiTrap) and an anion-exchange (5 mL HiTrap) column, pre-equilibrated with phosphate buffer (20 mM, pH 8.0). Chromatography was performed with isocratic elution and phosphate buffer (20 mM, pH 8.0) at a flow rate 2.0 mL/min. The active fractions were pooled for further study. All purification steps were performed at ambient temperature (25 °C).

Colloidal chitin preparation

Chitin powder (100 g) was suspended in concentrated HCl (600 mL) and incubated at room temperature until chitin powder was completely dissolved (about 3 h). The resulting solution was then poured into doubly deionized water (4 L) with rapid stirring to form the precipitant of colloidal chitin, which was subsequently collected by centrifugation at 7000 xg for 10 min at 4 °C. The precipitant was washed with sterile distilled water (200 mL each) several times to bring the pH value of the colloidal chitin suspension to 2.0-3.0. After neutralization with sodium hydroxide (1 M), the low-salt colloidal chitin was obtained on repeated centrifugation (7000 xg, 10 min) and washing with water 2-3 times. The acid-treated chitin was kept at 4 °C as a pellet for further applications.

Enzyme activity assay

Chitinase activity was measured via estimating the reducing ends of sugars. The assay was performed by mixing colloidal chitin (0.25 mL, 1%, pH 7.0), and suitably diluted enzyme (0.25 mL) for 1 h at 37 °C. The catalytic reaction was terminated and analyzed on adding dinitrosalicylic acid reagent[12] (0.5 mL). The mixture was boiled for 15 min, chilled and centrifuged to remove insoluble chitin. The resulting adduct of reducing sugars were measured spectrophotometrically at 540 nm. One unit of chitinase activity is defined as the amount of enzyme required to release detect-

able reducing sugars (1 μmol) in 1 min at 37 °C.

Electrospray mass spectrometric (ESI-MS) analysis

Mass spectra were recorded with a quadruple time-of-flight mass spectrometer (Q-TOF, Micromass, UK). The quadruple mass analyzer was scanned over a range 50-1500 u/*z* of mass-to-charge ratio for product analysis and 500-3000 for protein analysis, with a scan step 2 s and a inter scan 0.1 s/step.

Large-scale preparation of *N,N*-diacetyl chitobiose

The enzymatic degradation of colloidal chitin was performed in a fermenter (5 L) on adding chitinase (200 mL, 230 unit/mL, 20 mM NaOAc, pH 5.5) to colloidal chitin (4 L, 2.5%). The resulting pH value was measured to be 5.7. After reaction for 10 days, the resulting solution was centrifuged to remove the un-reacted chitin. The supernatant was concentrated to form highly viscous syrup with a rotary evaporator. 500 mL of ethanol (95%, w/w) was then added and stirred thoroughly to precipitate salt and protein, which were subsequently removed on centrifugation. After removal of ethanol, the *N,N*-diacetyl chitobiose formed as viscous syrup was stored at 4 °C for crystallization.

RESULTS AND DISCUSSION

PCR cloning and sequence of chitinase A from *S. marcescens*

Based on the *S. marcescens chi*A gene sequence, two oligonucleotides were designed and used as primers for PCR cloning. With the chromosomal DNA of *S. marcescens* as template, a DNA fragment (1.7 kb) was amplified and inserted into QuanTox and further constructed in pRSET for protein expression. The amplified *chi*A gene was confirmed with DNA sequencing. Sequence analysis (Fig. 1) revealed that the amplified *chi*A gene contains an open reading frame of 1692 bp encoding 563 amino-acid residues with the first 23 amino acids as signal peptide. As compared with other *chi*A genes deposited in Genbank, the deduced amino-acid sequence of this ChiA exhibited 7-26 residues in variation (data not shown), corresponding to >95 identity to other ChiAs.

Purification of recombinant chitinase A

The full *chi*A gene encoding the protein of *S. marcescens* chitinase A was sub-cloned into pRSET A. The resulting plasmid was transformed into the *E. coli* BL21 (DE3) strain, and cultivated in LB medium (37 °C, pH 7.0). The recombinant enzyme, expressed as a soluble form, was further isolated to become highly homogeneous with purification by hydrophobic interaction chromatography (HIC) (Fig. 2) followed with a unique column system coupled with both HiTrap SP (cationic exchange) and HiTrap Q (anion exchange) in series. The recombinant ChiA was eluted at a small salt concentration and at a non-binding region (0 mM NaCl), after which purification was performed using HIC and an ion-exchange column, successively and respectively. ChiA clearly possesses a hydrophobic surface. The final yield was 35%; 50-fold purification was achieved. SDS-PAGE analysis revealed that the recombinant ChiA was obtained with great homogeneity (> 90%). The estimated molecular mass was ~60 kDa. ESI-MS analysis gave a precise measurement, a molecular mass 58,607 Da (as shown in Fig. 3b), consistent with a theoretical calculation of the deduced protein without signal peptide.

Characteristics of the purified chitinase

The assay of pH-dependent and temperature-dependent activities showed that the recombinant ChiA had optimum activity near pH 7.0 and 45 °C (data not shown). The stability of the enzyme to pH and temperature was investigated with colloidal chitin as the substrate. In general, the enzyme was stable for pH 4.0-9.0 over 4 h; throughout the tested pH range the enzyme activity retained at least 80% the activity at pH 7.0. Although recombinant ChiA is pH-resistant, it is somewhat sensitive to temperature. An investigation of thermal stability showed that ChiA was stable up to 45 °C with incubation (4 h, pH 7.0); the activity decreased significantly for a temperature greater than 50 °C (Fig. 4). The catalytic outcomes of this recombinant enzyme are comparable to other reported ChiA.[13,14]

Product specificity

To optimize the catalytic reaction, we analyzed with ESI-MS the product of enzymatic hydrolysis of colloidal chitin obtained from the reaction under varied buffer system, including NaOAc (20 mM), phosphate or Tris, at varied acidity (pH 4-9). In general, the enzymatic products were a mixture of *N*-acetyl glucosamine and *N,N*-diacetyl chitobiose (Fig. 5a) when reactions were performed in phos-

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1       ATG  CGC  AAA  TTT  AAT  AAA  CCG  CTG  TTG  GCG  CTG  TTG  ATC  GGC  AGC
1       Met  Arg  Lys  Phe  Asn  Lys  Pro  Leu  Leu  Ala  Leu  Leu  Ile  Gly  Ser

46      ACG  CTG  TGT  TCC  GCG  GCG  CAG  GCC  GCC  GCG  CCG  GGC  AAG  CCG  ACC
16      Thr  Leu  Cys  Ser  Ala  Ala  Gln  Ala  Ala  Ala  Pro  Gly  Lys  Pro  Thr

91      ATC  GCC  TGG  GGC  AAC  ACC  AAG  TTC  GCC  ATC  GTT  GAA  GTT  GAC  CAG
31      Ile  Ala  Trp  Gly  Asn  Thr  Lys  Phe  Ala  Ile  Val  Glu  Val  Asp  Gln

136     GCG  GCT  ACC  GCT  TAT  AAT  AAT  TTG  GTG  AAG  GTA  AAA  AAT  GCC  GCC
46      Ala  Ala  Thr  Ala  Tyr  Asn  Asn  Leu  Val  Lys  Val  Lys  Asn  Ala  Ala

181     GAT  GTT  TCC  GTC  TCC  TGG  AAT  TTA  TGG  AAT  GGC  GAC  GCG  GGC  ACG
61      Asp  Val  Ser  Val  Ser  Trp  Asn  Leu  Trp  Asn  Gly  Asp  Ala  Gly  Thr

226     ACG  GCC  AAG  ATT  TTA  TTA  AAT  GGT  AAA  GAG  GCG  TGG  AGT  GGT  CCT
76      Thr  Ala  Lys  Ile  Leu  Leu  Asn  Gly  Lys  Glu  Ala  Trp  Ser  Gly  Pro

271     TCA  ACC  GGA  TCT  TCC  GGT  ACG  GCG  AAT  TTT  AAA  GTG  AAT  AAA  GGC
91      Ser  Thr  Gly  Ser  Ser  Gly  Thr  Ala  Asn  Phe  Lys  Val  Asn  Lys  Gly

316     GGC  CGT  TAT  CAA  ATG  CAG  GTG  GCA  TTG  TGC  AAT  GCC  GAC  GGC  TGC
106     Gly  Arg  Tyr  Gln  Met  Gln  Val  Ala  Leu  Cys  Asn  Ala  Asp  Gly  Cys

361     ACC  GCC  AGT  GAC  GCC  ACC  GAA  ATT  GTG  GTG  GCC  GAC  ACC  GAC  GGC
121     Thr  Ala  Ser  Asp  Ala  Thr  Glu  Ile  Val  Val  Ala  Asp  Thr  Asp  Gly

406     AGC  CAT  TTG  CCG  CCG  TTG  AAA  GAG  CCG  CTG  CTG  GAA  AAG  AAT  AAA
136     Ser  His  Leu  Pro  Pro  Leu  Lys  Glu  Pro  Leu  Leu  Glu  Lys  Asn  Lys

451     CCG  TAT  AAA  CAG  AAC  TCC  GGC  AAA  GTG  GTC  GGT  TCT  TAT  TTC  GTC
151     Pro  Tyr  Lys  Gln  Asn  Ser  Gly  Lys  Val  Val  Gly  Ser  Tyr  Phe  Val

496     GAG  TGG  GGC  GTT  TAC  GGG  CGC  AAT  TTC  ACC  GTC  GAC  AAG  ATC  CCG
166     Glu  Trp  Gly  Val  Tyr  Gly  Arg  Asn  Phe  Thr  Val  Asp  Lys  Ile  Pro

541     GCG  CAA  AAC  CTG  ACC  CAC  CTG  CTG  TAC  GGC  TTT  ATC  CCG  ATC  TGC
181     Ala  Gln  Asn  Leu  Thr  His  Leu  Leu  Tyr  Gly  Phe  Ile  Pro  Ile  Cys

586     GGC  GGC  AAT  GGC  ATC  AAC  GAC  AGC  CTG  AAA  GAG  ATT  GAA  GGC  AGC
196     Gly  Gly  Asn  Gly  Ile  Asn  Asp  Ser  Leu  Lys  Glu  Ile  Glu  Gly  Ser

631     TTC  CAG  GCG  TTG  CAG  CGC  TCC  TGC  CAA  GGC  CGC  GAG  GAC  TTC  AAA
211     Phe  Gln  Ala  Leu  Gln  Arg  Ser  Cys  Gln  Gly  Arg  Glu  Asp  Phe  Lys

676     ATC  TCG  ATC  CAC  GAT  CCG  TTC  GCC  GCG  CTG  CAA  AAG  GCG  CAG  AAG
226     Ile  Ser  Ile  His  Asp  Pro  Phe  Ala  Ala  Leu  Gln  Lys  Ala  Gln  Lys

721     GGC  GTG  ACC  GCC  TGG  GAT  GAC  CCC  TAC  AAG  GGC  AAC  TTC  GGC  CAG
241     Gly  Val  Thr  Ala  Trp  Asp  Asp  Pro  Tyr  Lys  Gly  Asn  Phe  Gly  Gln

766     CTG  ATG  GCG  CTG  AAG  CAG  GCG  CAT  CCT  GAC  CTG  AAA  ATC  CTG  CCG
256     Leu  Met  Ala  Leu  Lys  Gln  Ala  His  Pro  Asp  Leu  Lys  Ile  Leu  Pro

811     TCG  ATC  GGC  GGC  TGG  ACG  CTG  TCC  GAC  CCG  TTC  TTC  TTC  ATG  GGC
271     Ser  Ile  Gly  Gly  Trp  Thr  Leu  Ser  Asp  Pro  Phe  Phe  Phe  Met  Gly
856     GAC  AAG  GTG  AAG  CGC  GAT  CGC  TTC  GTC  GGT  TCG  GTG  AAA  GAG  TTC
286     Asp  Lys  Val  Lys  Arg  Asp  Arg  Phe  Val  Gly  Ser  Val  Lys  Glu  Phe
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901     CTG  CAG  ACC  TGG  AAG  TTC  TTC  GAC  GGC  GTG  GAT  ATC  GAC  TGG  GAG
301     Leu  Gln  Thr  Trp  Lys  Phe  Phe  Asp  Gly  Val  Asp  Ile  Asp  Trp  Glu

946     TTC  CCG  GGC  GGC  AAA  GGC  GCC  AAC  CCT  AAC  CTG  GGC  AGC  CCG  CAA
316     Phe  Pro  Gly  Gly  Lys  Gly  Ala  Asn  Pro  Asn  Leu  Gly  Ser  Pro  Gln

991     GAC  GGG  GAA  ACC  TAT  GTG  CTG  CTG  ATG  AAG  GAG  CTG  CGG  GCG  ATG
331     Asp  Gly  Glu  Thr  Tyr  Val  Leu  Leu  Met  Lys  Glu  Leu  Arg  Ala  Met

1036    CTG  GAT  CAG  CTG  TCG  GCG  GAA  ACC  GGC  CGC  AAG  TAT  GAG  CTG  ACC
346     Leu  Asp  Gln  Leu  Ser  Ala  Glu  Thr  Gly  Arg  Lys  Tyr  Glu  Leu  Thr

1081    TCC  GCC  ATC  AGC  GCC  GGT  AAG  GAC  AAG  ATC  GAC  AAG  GTG  GCT  TAC
361     Ser  Ala  Ile  Ser  Ala  Gly  Lys  Asp  Lys  Ile  Asp  Lys  Val  Ala  Tyr

1126    AAC  GTT  GCG  CAG  AAC  TCG  ATG  GAT  CAC  ATC  TTC  CTG  ATG  AGC  TAC
376     Asn  Val  Ala  Gln  Asn  Ser  Met  Asp  His  Ile  Phe  Leu  Met  Ser  Tyr

1171    GAC  TTC  TAT  GGC  GCC  TTC  GAT  CTG  AAG  AAC  CTG  GGG  CAT  CAG  ACC
391     Asp  Phe  Tyr  Gly  Ala  Phe  Asp  Leu  Lys  Asn  Leu  Gly  His  Gln  Thr

1216    GCG  CTG  AAT  GCG  CCG  GCC  TGG  AAG  CCG  GAC  ACC  GCT  TAC  ACC  ACG
406     la   Leu  Asn  Ala  Pro  Ala  Trp  Lys  Pro  Asp  Thr  Ala  Tyr  Thr  Thr

1261    GTG  AAC  GGC  GTG  AAT  GCG  CTG  CTG  GCG  CAG  GGC  GTC  AAG  CCG  GGC
421     Val  Asn  Gly  Val  Asn  Ala  Leu  Leu  Ala  Gln  Gly  Val  Lys  Pro  Gly

1306    AAA  ATC  GTC  GTC  GGC  ACC  GCC  ATG  TAT  GGC  CGC  GGC  TGG  ACC  GGG
436     Lys  Ile  Val  Val  Gly  Thr  Ala  Met  Tyr  Gly  Arg  Gly  Trp  Thr  Gly

1351    GTG  AAC  GGC  TAC  CAG  AAC  AAC  ATT  CCG  TTC  ACC  GGC  ACC  GCC  ACC
451     Val  Asn  Gly  Tyr  Gln  Asn  Asn  Ile  Pro  Phe  Thr  Gly  Thr  Ala  Thr

1396    GGG  CCG  GTT  AAA  GGC  ACC  TGG  GAG  AAC  GGC  ATC  GTG  GAC  TAC  CGC
466     Gly  Pro  Val  Lys  Gly  Thr  Trp  Glu  Asn  Gly  Ile  Val  Asp  Tyr  Arg

1441    CAA  ATC  GCC  AGC  CAG  TTC  ATG  AGC  GGC  GAG  TGG  CAG  TAT  ACC  TAC
481     Gln  Ile  Ala  Ser  Gln  Phe  Met  Ser  Gly  Glu  Trp  Gln  Tyr  Thr  Tyr

1486    GAC  GCC  ACG  GCG  GAG  GCG  CCT  TAC  GTG  TTC  AAA  CCT  TCC  ACC  GGC
496     Asp  Ala  Thr  Ala  Glu  Ala  Pro  Tyr  Val  Phe  Lys  Pro  Ser  Thr  Gly

1531    GAT  CTG  ATC  ACC  TTC  GAC  GAT  GCC  CGC  TCG  GTG  CAG  GCT  AAA  GGC
511     Asp  Leu  Ile  Thr  Phe  Asp  Asp  Ala  Arg  Ser  Val  Gln  Ala  Lys  Gly

1577    AAG  TAC  GTG  CTG  GAT  AAA  CAG  CTG  GGC  GGC  CTG  TTC  TCC  TGG  GAG
526     Lys  Tyr  Val  Leu  Asp  Lys  Gln  Leu  Gly  Gly  Leu  Phe  Ser  Trp  Glu

1621    ATC  GAC  GCG  GAC  AAC  GGC  GAT  ATT  CTC  AAC  AGC  ATG  AAC  GCC  AGC
541     Ile  Asp  Ala  Asp  Asn  Gly  Asp  Ile  Leu  Asn  Ser  Met  Asn  Ala  Ser

1666    CTG  GGC  AAC  AGC  GCC  GGC  GTT  CAA  TAA
556     Leu  Gly  Asn  Ser  Ala  Gly  Val  Gln  ***
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Fig. 1. DNA and amino-acid sequence of chitinase A cloned from *Serratia marcescens*. The first 23 residues are a signal peptide (underlined).

phate buffer (pH 6-8), Tris buffer (pH 7.5-9) or $NaHCO_3$ (pH 9.0). However, *N,N*-diacetyl chitobiose is the predominant product for a NaOAc buffer with pH in a range 4-6. The product specificity is unlikely controlled by acidity as both *N*-acetyl glucosamine and disaccharide were produced when NH_4OAc (pH 5-6) was used as buffer. Although the catalytic detail for product specificity of *S. marcescens* ChiA is unclear, our finding might yield an improved clue for the inconsistency of product specificity reported in the literature.[15-19] Taking into account the buffer effect on product specificity and enzyme activity, we chose NaOAc (20 mM, pH 5.5) as the buffer system for the large-scale production of *N,N*-diacetyl chitobiose.

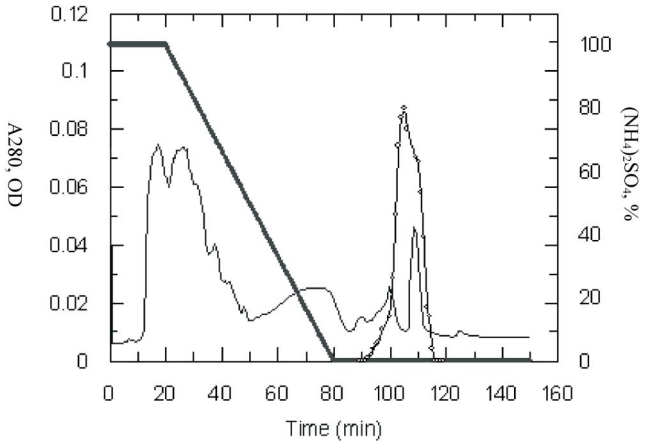

Fig. 2. Purification of ChiA by hydrophobic interaction chromatography. The column was pre-equilibrated with ammonium sulfate buffer (1 M, pH 7.0). Elution with linear gradient of ammonium sulfate (heavy line) was monitored at 280 nm (light line) and the enzyme activity (-o-) was measured.

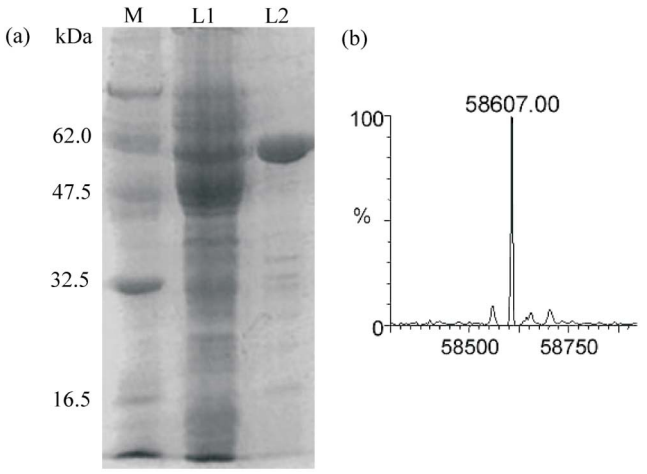

Fig. 3. SDS-PAGE and ESI-MS analyses of purified chitinase. (a) Molecular mass estimated from SDS-PAGE analysis is ~60 kDa. M: protein marker; L1: crude enzyme from intracellular; L2: ChiA purified by a series of ion-exchange column. (b) ESI-MS analysis showed that the molecular mass of purified ChiA is 58608 Da.

Large-scale preparation of *N,N*-diacetyl chitobiose

A batch reaction (scale 100 g) was performed on adding enzyme (75 mg) to a colloidal chitin solution (4 L, 2.5%) with NaOAc (pH 5.5, 20 mM). The reaction was periodically monitored with DNS assay and ESI-MS analysis for 10 d at 37 °C. At the end of the reaction, a light yellowish solution was obtained. After concentration, the viscous solution was re-suspended in ethanol. The insoluble salt and protein were largely removed and the *N,N*-diacetyl chitobiose was satisfactorily soluble in ethanol (95%). *N,N*-diacetyl chitobiose (80 g, corresponding to 80% yield mass/mass) was obtained. The final product was analyzed with TLC and ESI-MS. Only a clear spot, corresponding to *N,N*-diacetyl chitobiose, was observed with TLC analysis (Fig. 5a, lane 2). ESI-MS analysis showed a similar result. As shown in Fig. 5b, with little contamination by GlcNAc (m/z = 244, GlcNAc + Na^+), the predominant product is *N,N*-diacetyl chitobiose. The signals at m/z = 447 and 871 were identified as *N,N*-diacetyl chitobiose + Na^+ and 2(*N,N*-diacetyl chitobiose) + Na^+, respectively.

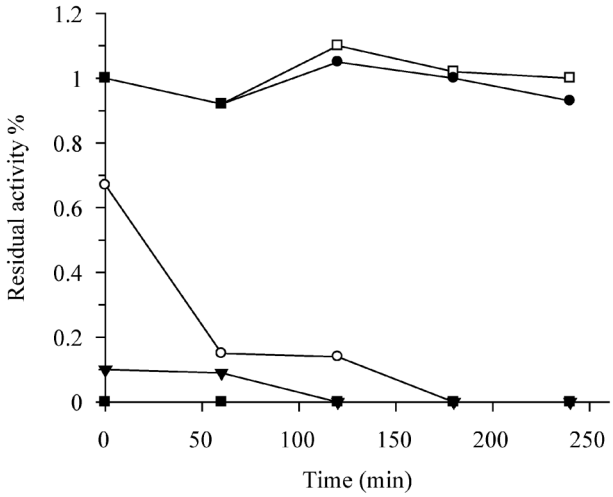

Fig. 4. Thermal stability of ChiA. The residual activity monitored at 37 °C (□), 45 °C (•), 50 °C (o), 55 °C (▼), 60 °C (■).

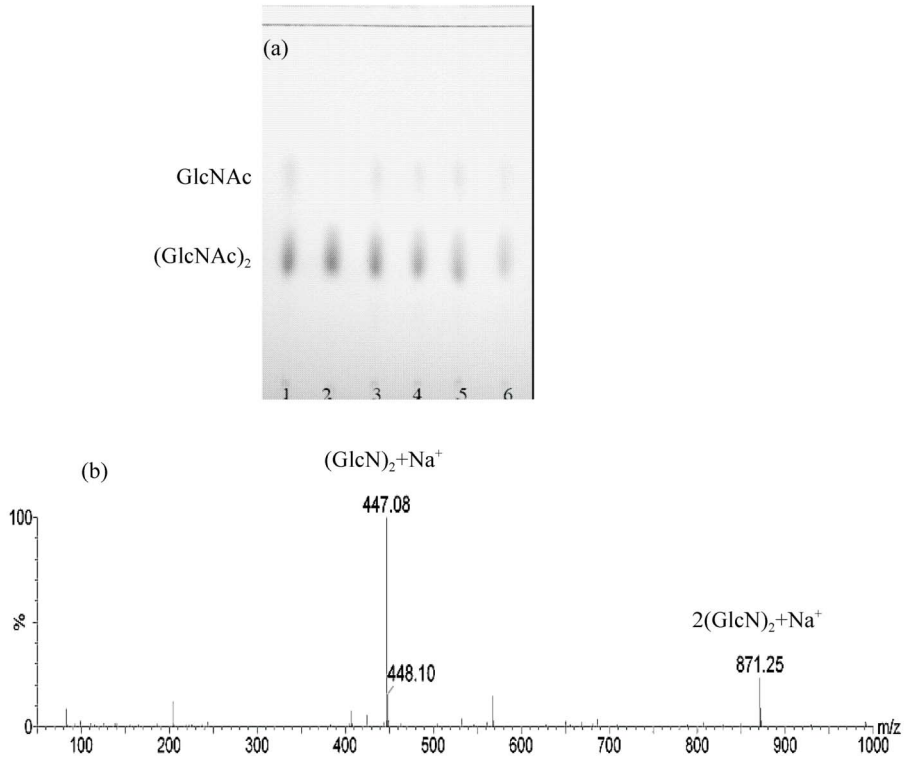

Fig. 5. Product analysis with (a) TLC and (b) ESI/MS. (a) Enzymatic product analysis by TLC. GlcNAc and $(GlcNAc)_2$ standard (Lane 1); products from enzymatic reaction with varied buffers: NaOAc, pH 5.5 (Lane 2); phosphate, pH 6.0 (Lane 3); phosphate, pH 7.0 (Lane 4); Tris, pH 8.0 (Lane 5); $NaHCO_3$, pH 9.0 (Lane 6), (b) ESI/MS analysis of the enzymatic product obtained from large-scale reaction with NaOAc (20 mM, pH 5.0).

CONCLUSION

N,*N*-diacetyl chitobiose is potentially useful for biological applications, but its availability and cost limit its further extensive research and applications. In this work, we established a feasible method for the preparation of *N*,*N*-diacetyl chitobiose as predominant product by using recombinant chitinase A of *S. marcescens*. The high-quality disaccharide produced in large-scale without column chromatographic separation is now possible.

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