1 **Cloning, characterization and substrate degradation mode of a novel chitinase**

2 **from *Streptomyces albolongus* ATCC 27414**

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19 **Abstract**

20 A novel chitinase gene was cloned from *Streptomyces albolongus* ATCC 27414,
21 and expressed successfully in *Escherichia coli* BL21. The recombinant enzyme
22 (SaChiA4) belongs to glycoside hydrolases (GH) family 18 and consists of a catalytic
23 domain and a chitin binding domain (CBD) in its C-terminus. SaChiA4 was purified
24 homogeneously (specific activity of 66.2 U/mg with colloidal chitin as substrate), and
25 showed a molecular mass of approximately 47 kDa. SaChiA4 showed its optimal
26 activity at pH 5.0 and 55 °C and exhibited remarkable pH and temperature stability.
27 SaChiA4 proved to have an higher specificity toward glycosides containing acetyl
28 groups and hydrolyzes the substrates in an non-processive manner with higher ability
29 to produce $(GlcNAc)_2$ and GlcNAc. The results indicated that SaChiA4 is a novel
30 endo-type chitinase, which has potential applications in the treatment of chitin wastes
31 and the production of $(GlcNAc)_2$.

32 **Keywords:** Chitinase; Cloning; Characterization; Substrate degradation mode;
33 *Streptomyces albolongus*

34

35 **1. Introduction**

36 Chitin is a highly insoluble linear biopolymer, composed of β-1,4-linked units of
37 N-acetyl-D-glucosamine (GlcNAc). It is the second most abundant natural
38 polysaccharide after cellulose, produced up to about 100 billion tons per year (Haki &
39 Rakshit, 2003). Chitin is the major component of exoskeletons of insects, shells of
40 crustaceans (such as shrimp and crab) and the cell walls of fungi (Ikeda, Kondo, &
41 Matsumiya, 2013; Kumar, 2000). In spite of such abundance, chitin has limited
42 applications, mainly because of its insolubility in water or organic solvents. Instead,
43 the degradation products of chitin, N-acetyl chitooligosaccharides (NCOSs) and free
44 GlcNAc, have received more attention because they have a variety of physiological
45 applications. For example, NCOSs can be used as the main carbon sources for the
46 growth of enteric beneficial microbiota (such as lactic acid bacteria and bifidobacteria)
47 and have been proved to exhibit antibacterial activity (Chen, Chang, Mau, & Yen,
48 2002). Consequently, NCOSs with low molecular weight can be used as functional
49 food ingredients or additives in the food industry (Ngo, Wijesekara, Vo, Ta, & Kim,
50 2011). Furthermore, GlcNAc has been widely used as functional food or beverage
51 supplement due to its antioxidation and immune-enhancing activity (Rogers, Fosdick,
52 & Bohlmann, 2004). Therefore, to produce NCOSs and GlcNAc with high yields and
53 high quality is of great importance in the food industry.

54 Traditional degradation of chitin relies on chemical methods that generate some
55 problems including environmental pollution generated by using chemical reagents
56 (e.g., sodium hydroxide and hydrochloric acid), low yield and purity of products and

57 the uncontrollability of the reaction process (Hammami, Siala, Jridi, Ktari, Nasri, &
58 Triki, 2013; Wang, Lin, Yen, Liao, & Chen, 2006). As a result, in recent years, the
59 environmentally friendly bioconversion of chitin by chitinases has become a process
60 of considerable importance.

61 Chitinases are glycoside hydrolytic enzymes that cleave the β-1,4 glycosidic
62 bonds. They have many potential applications in a wide range of fields such as the
63 production of NCOS or GlcNAc in food field (Aam, Heggset, Norberg, & Eijsink,
64 2010; Chen, Shen, & Liu, 2010), as antifungal agent against pathogenic organisms in
65 agriculture (S. L. Wang, Lin, Yen, Liao, & Chen, 2006). Furthermore, they find
66 applications for the utilization and clean-up of chitin wastes (e.g., shrimp shell wastes
67 from industrialized food processing), allowing environmental protection and the
68 conversion of chitin to bioethanol in the energy industry (Purushotham & Podile,
69 2012; Zhu, Wang, Liu, & Yang, 2016).

70 According to the position where the hydrolysis of chitin occurs, chitinases have
71 been grouped into endochitinases (EC 3.2.1.14), exochitinases (EC 3.2.1.29) and
72 N-acetylglucosaminidases (EC 3.2.1.30) (Cohenkupiec & Chet, 1998; Duochuan,
73 2006). Endochitinases randomly cleave the chitin chains to release soluble and
74 smaller weight oligomers of diverse size, exochitinases cut the β-1,4 glycosidic bonds
75 of chitin from the non-reducing end or reducing end to generate $(GlcNAc)_2$, and
76 N-acetylglucosaminidases catalyze the release of GlcNAc from NCOS or $(GlcNAc)_2$
77 (Ephraim, 2010; Hamid, et al., 2013). Based on amino acid conserved sequence,
78 chitinases are classified into glycoside hydrolases (GH) family 18 and GH family 19

79 in the Carbohydrate-Active enzymes database (CAZy)
80 (http://www.cazy.org/Glycoside-Hydrolases.html) (BL, PM, C, T, V, & B, 2009).
81 Chitinases are distributed in various living organisms. The GH-18 chitinases are
82 mainly produced by bacteria, fungi, insects, plants, vertebrates and mammals (Itoh, et
83 al., 2013; Lee, et al., 2007; Mander, et al., 2016). Compared with the GH-18
84 chitinases, most GH-19 chitinases have been found in higher plants, while some
85 bacteria have been found to secret GH-19 chitinases, such as *Streptomyces* sp
86 (Okazaki, Yamashita, Noda, Sueyoshi, Kameshita, & Hayakawa, 2004). So,
87 *Streptomyces* species are one of the good sources of chitinases.

88 *Streptomyces* sp., as a kind of gram-positive soil actinobacteria, are widely found
89 in nature. Several chitinases have been isolated from *Streptomyces* sp., in particular
90 from *Streptomyces roseolus* (Jiang, Chen, Hong, Wang, Chen, & Zou, 2012),
91 *Streptomyces anulatus* CS242 (Mander, et al., 2016), *Streptomyces coelicolor* A3(2)
92 (Nguyen-Thi, et al., 2016), *Streptomyces griseus* (Berger & Reynold, 1958), and
93 *Streptomyces glauciniger* WICC-A03 (Awad, El-Enshasy, Hanapi, Hamed, & Rosidi,
94 2014). However, to date, few chitinase has been cloned, expressed and isolated from *S.*
95 *albolongus* ATCC 27414. In this work, we successfully performed the cloning,
96 expression, biochemical characteristics and catalytic mechanism of a novel
97 endo-chitinase (SaChiA4) from *S. albolongus* ATCC 27414.

98 **2. Materials and methods**

99 *2.1. Materials*

100 Chitin was prepared from shrimp shells as previously described (Xue, et al.,

101 2014). Chitosan (with a degree of deacetylation (DD) > 90.0%) was purchased from
102 Shanghai Lanji technology development co., LTD (Shanghai, China). NCOS (the
103 degrees of polymerization (DP) is 1 to 6) was purchased from Qingdao BZ Oligo
104 Biotech (Qingdao, China). Ni-NTA Resin was purchased from TransGen Biotech
105 (Beijing, China). Carboxymethylcellulose sodium (CMC-Na) was obtained from
106 Tianjin Guangfu Fine Chemical Research Institude (Tianjin, China). Silica gel plate
107 was obtained from Qingdao Shenghai Fine Silicone Chemical Co, Ltd (Qingdao,
108 China). All other reagents used were of analytical grade unless otherwise specific
109 description.

110 *2.2. Sequence analysis*

111 The amino acid sequence of SaChiA4 was obtained via DNAMAN and analyzed
112 by BLAST at National Center for Biotechnology Information (NCBI), respectively.
113 Protein function prediction was performed by NCBI, and protein homologous
114 sequences alignment was carried out by ClustalX 2.1 and ESPript
115 (http://espript.ibcp.fr/ESPript/ESPript/). For phylogenetic tree analysis, MEGA 6.0
116 was used.

117 *2.3. Strains and culture conditions*

118 *S. albolongus* ATCC 27414, purchased originally from China General
119 Microbiological Culture Collection Center (CGMCC), was cultivated at 28 °C, in a
120 medium composed of 1% soluble starch and 0.2% yeast extract in distilled water with
121 pH 7.3. *Escherichia coli* DH5α and *Escherichia coli* BL21 (DE3) were grown at
122 37 °C, in Luria-Bertani (LB) medium containing 0.5% yeast extract, 1% tryptone and

123 1% NaCl.

124 *2.4. Preparation of colloidal chitin*

125 The preparation of substrate colloidal chitin was in accord with the method
126 described by Jianzhi Zhang (Zhang, Kopparapu, Yan, Yang, & Jiang, 2013), with
127 some subtle differences. Chitin was dissolved (5 g in 100 mL) with concentrated
128 hydrochloric acid (HCl) and placed at 4 °C for 24 h, after which the mixtures was
129 added to 300 mL of 50% ethanol stirred vigorously and followed by a centrifugation
130 step at 8000 ×g for 15 min at 4 °C. The precipitate was washed to neutral pH with
131 distilled water.

132 *2.5. Gene cloning and expression of SaChiA4*

133 The genomic DNA, used as the template of polymerase chain reaction (PCR),
134 was extracted from *S. albolongus* ATCC 27414, using puregene Yeast/Bact. Kit B
135 (QIAGEN, Beijing, China). Two primers SaChiA4F
136 (5'-CCGGAATTCATGGAACGCGTACTACCC-3') and SaChiA4R
137 (5'-CCCAAGCTTGCAGGCGCCGTTGTCCGC-3') (BGI, Shenzhen, China) with
138 *BamH*I and *Hind*III recognition cites, were synthesized for amplification of the
139 SaChiA4 gene. The PCR products were purified and ligated into the pET28a (+)
140 vector, which contains two 6 ×His tags. The recombinant were translated into
141 *Escherichia coli* DH5α, which were grown in solid LB medium with 50 μg/mL
142 kanamycin at 37 °C for 16 h. The positive clones called SaChiA4 were selected and
143 screened by PCR and sequencing to verify the correctness of the SaChiA4 nucleotide
144 sequence. The ultimate recombinant expression vectors was transformed into

145 *Escherichia coli* BL21 (DE3) for protein expression. *E. coli* BL21 containing
146 SaChiA4, were cultivated in a ZYP-5052 autoinduction medium (1.0% tryptone, 0.5%
147 yeast extract, 0.7% Na_2HPO4, 5%, 0.7% KH_2PO4, 0.3% $(NH4)_2SO4$, 0.05% $MgSO_4$,
148 0.5% glycerin, 0.05% glucose and 0.2% α-galactose), at 20 °C for 48 h in a shaker
149 with a rotation speed of 220 rpm.

150 *2.6. Purification of recombinant SaChiA4*

151 All purification steps were performed at 4 °C. The cells were harvested by
152 centrifugation at 8000 ×g for 10 min, after which the cell precipitation was
153 re-suspended with 50 mM Tris-HCl buffer (pH 7.4) and disrupted by sonication. Cell
154 debris were removed by centrifugation at 8000 ×g for 20 min at 4 °C and the
155 supernatant containing the crude enzyme was collected. Next, the crude enzyme was
156 loaded on a Ni-NTA column (8 × 1 cm), which was pre-equilibrated with 6 column
157 volumes buffer A (50 mM Tris-HCl, 5 mM imidazole and 500 mM NaCl), followed
158 by buffer B (50 mM Tris-HCl, 50 mM imidazole and 500 mM NaCl) to wash out the
159 weakly bound protein or impurities. The fractions showing high chitinase activity
160 were eluted with buffer C (50 mM Tris-HCl, 100 mM imidazole and 500 mM NaCl)
161 and concentrated using 30 kDa ultrafiltration device for determination of purity by
162 sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

163 *2.7. Enzyme activity assay and protein measurement*

164 SaChiA4 activity was determined by the method of Yang (Yang, Fu, Yan, Guo,
165 Liu, & Jiang, 2016) with some modification. The reaction system containing 195 μL
166 of 0.1g/mL colloidal chitin dissolved with citrate buffer (pH 5.0) and 5 μL of purified

167 enzyme (5 U/mL) was incubated at 55 °C for 30 min.

168 The reaction stopped by boiling for 10 min was added 300 μL 3,5-dinitrosalicylic 169 acid (DNS), followed by boiling for 10 min and then cooled immediately. Samples 170 were diluted by adding 1 mL water, and centrifuged at 10,000 ×g for 5 min. The 171 absorbance of supernatant was measured at 540 nm. One unit of SaChiA4 activity (U) 172 was defined as the amount of enzyme needed to liberate 1 μmol reducing sugars per 173 min under the enzyme assay conditions.

174 Specific activity of SaChiA4 was formulated by units per milligram protein. 175 Protein concentration was measured according to the Bradford method using bovine 176 serum albumin (BSA) as a standard (Bradford, 1976).

177 *2.8. Characterization of SaChiA4*

178 The optimum pH for activity of SaChiA4 was determined using different buffers 179 with 50 mM citrate buffer (pH 3.0 to pH 6.0), 50 mM phosphate buffer (pH 6.0 to pH 180 8.0), Tris-HCl buffer (pH 7.0 to pH 9.0) and glycine-NaOH buffer (pH 9.0 to pH 10.0) 181 at 55 °C for 30 min. To determine the pH stability, enzyme solutions were incubated 182 in above mentioned pH buffers at 25 °C for different time intervals, and then the 183 residual enzyme activities were examined under enzyme assay.

184 The optimum temperature of SaChiA4 activity was measured, and the reaction 185 solutions were incubated at temperatures that ranged from 30 to 80 °C for 30 min. To 186 determine the thermostability of SaChiA4, enzyme solutions were pre-incubated at 187 different temperatures ranging from 35 to 60 °C for different time intervals, the 188 remaining enzyme activities were investigated under the standard assay. All samples

were repeated three times.

The effects of various metal ions (Fe^{3+}, Ca^{2+}, Cu^{2+}, Mg^{2+}, Zn^{2+}, Mn^{2+}, Ni^{2+}, Ba^{2+}, Co^{2+}, K^{+}, Na^{+}) and chemical reagents (Na_2EDTA, Sodium dodecyl sulfate (SDS)) on SaChiA4 was investigated by adding individual reagents to enzyme solutions at final concentration of 1 mM and 10 mM which was incubated at 55 °C for 30 min.

2.9. Substrate specifity of SaChiA4

The substrate specifity of SaChiA4 was determined at 55 °C for 30 min in 50 mM citrate buffer with different substrates (1% w/v) including colloidal chitin, chitin powder, chitosan and CMC-Na.

2.10. Hydrolysis products and Degradation pattern analysis

The hydrolysis products of SaChiA4 were analyzed by High Performance Liquid Chromatography (HPLC). The reaction mixtures containing 198 μL substrates (0.1 g/mL colloidal chitin and 0.02 g/mL NCOS (DP 2-6)) and 2 uL of purified enzyme (5 U/mL) were incubated at 55 °C for different time intervals (0 min, 2 min, 15 min, 30 min, 60 min and 24 h), followed by boiling for 10 min to terminate the reaction. After centrifugation, the supernatant was loaded on a Sugar Pak I column（6.5 × 300 mm） which was re-equilibrated by 50 mg/mL Ethylenediaminetetraacetic Acid Disodium Calcium Salt at a flow speed of 0.2 mL/min for 12 h. The products were examined by Refractive Index Detector (RID) under the following conditions: the temperature of column was 75 °C, the mobile phase was 50 mg/mL $EDTA_2Na$ and the flow rate was 0.2 mL/min.

3. Result and discussion

211 *3.1. Sequence analysis of SaChiA4*

212 An entire opening reading frame (ORF) of SaChiA4 consisting of 1281 bp,
213 encodes a protein of 427 amino acid residues with a predicted molecular weight of 47
214 kDa and a predicted isoelectric point of 5.41. The GenBank accession number is
215 MG676685. Based on sequences alignment, SaChiA4 showed the highest sequence
216 identity (19.72%) with chitinase (Chain A) from *Pyrococcus furiosus* (2DSK_A)
217 (Nakamura, Mine, Hagihara, Ishikawa, & Uegaki, 2006), followed by sequence
218 similarity of 16.40% with chitinase from *Ewingella americana* (CAA62151.1) which
219 belong to GH family 18. Thus, the phylogenetic tree indicated that the SaChiA4 was a
220 new member of GH family 18 (Fig.1A). According to the CD-Search of NCBI,
221 SaChiA4 is composed of two functional domains: a catalytic domain and a chitin
222 binding domain (CBD) in its C-terminus. The catalytic domain was ranged from
223 residues 59 to 341. Conserved glutamate (Glu) residues were discovered in the
224 catalytic domains of SaChiA4, which were considered as proton donors to protonate
225 the glycosidic oxygen and played an important role in catalytic reactions (Fig.1B)
226 (Lienemann, Boer, Paananen, Cottaz, & Koivula, 2009). The CBD was from residues
227 393 to 423. Among GH family 18 chitinases, some aromatic residues were conserved
228 in chitin binding domains, which were deemed to be significant to facilitate the
229 hydrolysis of insoluble chitin (Shen & Jacobslorena, 1999; Uchiyama, Katouno,
230 Nikaidou, Nonaka, Sugiyama, & Watanabe, 2001). And in the CBD of SaChiA4,
231 tryptophan (Trp) and tyrosine (Tyr) residues were well conserved (Fig.1C) (Jiang, et
232 al., 2012). In addition, between catalytic domain and CBD, some proline residues as

233 well as several serine and threonine residues in chitinase from *Bacillus* sp. DAU101
234 were also found, which were conserved in serine/threonine (S/T)-rich linkers (Lee, et
235 al., 2007). In general, on the one hand, the catalytic domain and CBD are connected
236 via a (S/T)-rich linker (Arakane, Zhu, Matsumiya, Muthukrishnan, & Kramer, 2003;
237 Tomme, Warren, & Gilkes, 1995), on the other hand, the (S/T)-rich linkers could act
238 as receptor cites for glycosylation to assist in bracing chitinases and prevent the
239 hydrolysis of protease (Huang, et al., 2012). The results indicated that SaChiA4 is a
240 novel chitinase which belongs to GH family 18.

241 *3.2. Expression and purification of SaChiA4*

242 The full length gene of SaChiA4 from *S. albolongus* ATCC 27414 without the
243 terminator codon (TGA) was cloned and successfully expressed in *E. coli* BL21 as an
244 active protein with two His tags containing a N-terminal and a C-terminal His tag.
245 The recombinant SaChiA4 was purified by a Ni-NTA column and the purity and
246 molecular mass was detected by SDS-PAGE. A single homogeneous band appeared
247 on SDS-PAGE clearly and the molecular mass was estimated to be approximately 47
248 kDa, corresponding to that predicted by amino acid sequences. The purified protein
249 was specified as SaChiA4 （Fig. 2）.

250 The specific activity of purified SaChiA4 with colloidal chitin as substrate was
251 66.2 U/mg and after purification, the recovery rate was 32.5%. The specific activity
252 was higher than that observed for other previously reported chitinases expressed
253 heterologously in *E. coli* BL21, including Chib from *Bacillus* sp. DAU101 (0.73
254 U/mg) (Lee, et al., 2007), PbChi70 from *Paenibacillus barengoltzii* (30.0 U/mg)

255 (Yang, et al., 2016) and ChiIII from *Coprinopsis cinerea* (0.05 U/mg) (Niu, et al.,
256 2016).

257 *3.3. Effects of pH and temperature on activity and stability of SaChiA4*

258 SaChiA4 had its highest activity at pH 5.0 in 50 mM citrate buffer using
259 colloidal chitin as substrate, and more than 70% of its highest activity was showed in
260 a range of pH 4.0 to 6.0 (Fig. 3A). After preincubation for 72 h at 25 °C without
261 substrate, more than 50% of residual activities were retained within a broad pH range
262 from pH 3.0 to 9.0, and after incubation for 24 h, more than 60% residual activity was
263 retained (Fig. 3B). The result suggested that the SaChiA4 had a remarkable pH
264 stability. The optimum temperature of SaChiA4 was determined to be 55 °C, and
265 SaChiA4 showed more than 70% of its maximum activity at a wide range of
266 temperature from 35 to 65 °C (Fig. 3C). After incubation of SaChiA4 for 50 h in 50
267 mM citrate buffer without substrate, more than 50% of residual activities were
268 maintained at a range of temperature from 35 to 55 °C, and after incubation for 24h,
269 SaChiA4 still had more than 60% of its highest activity (Fig. 3D). The optimum pH
270 and temperature of SaChiA4 were similar to some other chitinases from bacteria or
271 fungus such as *Paenibacillus barengoltzii* (Yang, et al., 2016), *Paenibacillus.* Sp D1
272 (Singh & Chhatpar, 2011), *Streptomyces anulatus* CS242 (Mander, et al., 2016), and
273 *Paecilomyces thermophile* (Kopparapu, Zhou, Zhang, Yan, Liu, & Jiang, 2012),
274 whereas its pH stability and thermostability were superior to that of above chitinase.

275 *3.4. Effects of chemical agents on chitinase activity.*

276 SaChiA4 activity was inhibited by Fe^{3+}, Cu^{2+}, Na_2EDTA and SDS, and the

277 higher the concentration was, the stronger the inhibition power was. Besides, 1mM
278 and 10 mM SDS strongly inhibit the enzyme activity by 35% and 23%, respectively.
279 Mn^{2+}, Ba^{2+}, Na^{+} slightly increased SaChiA4 activity when used at low (1 mM)
280 concentration. At higher concentration (10 mM) the activation effect remained
281 constant with Mn^{2+}, but it was weakened for the other two metal ions. Other metal
282 ions slightly affected the enzyme activity (Table 1).

283 *3.5. Substrate specific of SaChiA4*

284 SaChiA4 exhibited its higher activity toward colloidal chitin than toward chitin
285 powder and chitosan (27.48% and 34% of colloidal chitin, respectively). This
286 difference may be because chitin powder has more and stronger crystal structures, and
287 for chitosan, the contents of acetyl components were only a small part. SaChiA4
288 hardly hydrolyze CMC-Na (Table 2). Therefore, SaChiA4 showed strict substrate
289 specifity, and it may be more specific to the chitin chains of β-1,4 linked
290 GlcNAc-GlcNAc, GlcNAc-GlcN or GlcN-GlcNAc.

291 *3.6. Hydrolysis products of colloidal chitin by SaChiA4*

292 To detect the final enzymatic hydrolysis products of chitin, the reaction medium
293 was analyzed by HPLC at different times. SaChiA4 efficiently degraded colloidal
294 chitin and $(GlcNAc)_2$ and small amount of $(GlcNAc)_3$ were observed after 2 min, and
295 the concentrates of $(GlcNAc)_2$ and $(GlcNAc)_3$ were 0.12 and 0.02 mg/mL,
296 respectively. And as the reaction proceeded for 30 min, the $(GlcNAc)_3$ were exhausted
297 (no detectable amount was found) and the $(GlcNAc)_2$ (0.78 mg/mL) rapidly increased,
298 together with small amount of GlcNAc (0.13 mg/mL). After hydrolysis of 24 h, the

299 content of $(GlcNAc)_2$ reached 2.17 mg/mL and the content of GlcNAc was 0.87
300 mg/mL, but with no other chitooligosaccharides (Fig. 4A and Fig. 4B). Therefore, the
301 result revealed that the end products of chitin by SaChiA4 were $(GlcNAc)_2$ and
302 GlcNAc, in which, $(GlcNAc)_2$ was dominant. The hydrolysis products of SaChiA4
303 were similar to some reported chitinases, such as Chisb from *Bacillus* sp. DAU101
304 (Lee, et al., 2007) and PbChi70 (Yang, et al., 2016) from *Paenibacillus barengoltzii*.
305 However, these two chitinases share only a sequence identity of 11.06% and 9.91%
306 with the sequence of SaChiA4. These features indicated that SaChiA4 is a novel
307 biocatalyst which has potential industrial applications for bioconversion of chitin
308 wastes and the production of valuable $(GlcNAc)_2$ and GlcNAc.

309 *3.7. Degradation pattern analysis of SaChiA4*

310 Chitinases have an active catalytic site, cleaving long-chain polymers of chitin or
311 NCOS in a processive or non-processive manner. In general, exo-chitinases cleave its
312 substrates in a processive manner to release $(GlcNAc)_2$ or GlcNAc from the reducing
313 or non-reducing end of the chain, for which, after a hydrolytic reaction, the substrate
314 would slide along the binding pocket for the next reaction (Nguyen-Thi et al., 2016).
315 Endo-chitinases generally degrade the chains of substrates randomly, for which, the
316 substrates and enzymes are separate after last reaction cycle (Horn, et al., 2006).

317 To investigate the degradation pattern of SaChiA4, the products of course-time
318 hydrolysis with some N-acetyl chitooligosaccharides (DP 2 to 6) as substrates were
319 analyzed by HPLC. As shown in Fig.4, $(GlcNAc)_3$ were converted to $(GlcNAc)_2$ and
320 GlcNAc (Fig. 4D). $(GlcNAc)_4$ were converted to $(GlcNAc)_2$ in the begining of the

321 reaction (after 2 min). After 15 min, a small amount of $(GlcNAc)_3$ accumulated and
322 $(GlcNAc)_2$ increased quickly. With the prolongation of reaction time, $(GlcNAc)_4$ and
323 $(GlcNAc)_3$ were further converted to $(GlcNAc)_2$ (Fig. 4E). For $(GlcNAc)_5$, SaChiA4
324 initially degraded it to yield $(GlcNAc)_3$ and $(GlcNAc)_2$ with the release of a trace
325 amount of $(GlcNAc)_4$ and GlcNAc, which were almost invisible in Figure, and as the
326 incubation was proceeded, the $(GlcNAc)_4$ and $(GlcNAc)_3$ were degraded into
327 $(GlcNAc)_2$ and GlcNAc (Fig. 4F). In terms of the degradation process of $(GlcNAc)_6$,
328 $(GlcNAc)_6$ were converted to $(GlcNAc)_2$, (GlcNAc)4 and $(GlcNAc)_3$ at initial time.
329 After 60min of reaction, the $(GlcNAc)_4$ were almost completely hydrolyzed to
330 $(GlcNAc)_2$, and with the extension of incubation time, the ultimate products were
331 $(GlcNAc)_2$ and GlcNAc (Fig. 4G). Since the appearance of odd-numbered of sugar at
332 the initial stage, SaChiA4 may act in a non-processive manner. For $(GlcNAc)_2$,
333 SaChitA4 hardly degrade it (Fig. 4C). So, for SaChitA4, the smallest unit of action is
334 $(GlcNAc)_3$, and the main products of NCOS were $(GlcNAc)_2$ and GlcNAc.

335 To further elucidate the mode of action, the calculated products of initial
336 time-course degradation of $(GlcNAc)_6$ by five-fold diluted SaChiA4 was showed in
337 Fig. 4H, and the result showed that $(GlcNAc)_6$ produced almost equal amounts of
338 $(GlcNAc)_4$ and $(GlcNAc)_2$ at the initial time of the reaction. Therefore, we confirm
339 that SaChiA4 is a novel endo-type chitinase, and there are some reports about
340 endo-chitnases at present, but some of which cannot degrade $(GlcNAc)_4$ or $(GlcNAc)_3$
341 (Ikeda, Kondo, & Matsumiya, 2013; Nguyen-Thi & Doucet, 2016). SaChiA4 is
342 capable of cleaving short-chain polymers and has a high ability to generate

343 $(GlcNAc)_2$.

344 **4. Conclusions**

345 In conclusion, we reported a novel endo-type chitinase (SaChiA4) that belongs to
346 GH18 family. SaChiA4 was purified and characterized with remarkable pH stability,
347 temperature stability and higher specific activity. It has relatively strict substrate
348 specificity and could efficiently degraded the colloidal chitin in a non-processive
349 manner to produce purified $(GlcNAc)_2$ and GlcNAc, which can be utilized in food or
350 pharmaceutical industries.

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355

356 **Abbreviations:**

357 CBD: chitin binding domain; GlcNAc: N-acetyl-D-glucosamine; $(GlcNAc)_2$:
358 N-acetyl chitobiose; NCOSs: N-acetyl chitooligosaccharides; GH: glycoside
359 hydrolases; DD: degree of deacetylation; DP: degree of polymerization; CMC-Na:
360 Carboxymethylcellulose sodium; HPLC: High Performance Liquid Chromatography;
361 LB: Luria-Bertani; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel
362 electrophoresis; DNS: 3,5-dinitrosalicylic acid; BSA: bovine serum albumin; RID:
363 Refractive Index Detector; ORF: opening reading frame.

364

365

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489

490

491 **Figure legends**

492 **Fig. 1.** (A) Phylogenetic analysis of SaChiA4 and other GH18 chitinases, based on
493 their amino acid sequences, displayed as a neighbor-joining tree. Each sequence is
494 marked with protein accession numbers, enzyme names and organisms. Branch
495 support is indicated by bootstrap confidence values (the numbers on the branches).
496 The scale bar indicated 0.2 substitutions per site. (B) Sequence alignment of partial
497 predicted catalytic domain of SaChiA4 with other GH18 chitinases. The listed
498 sequences include the chitinases from *Aeromonas* sp. 10S-24 (BAA06605.1),
499 *Pyrococcus furiosus* (2DSK_A), *Ewingella Americana* (CAA62151.1), *Bacillus sp*.
500 DAU101 (ABC66095.1) and *Eisenia Andrei* (ALG76037.1). Red triangle indicates
501 conserved amino acid residue (Glu). (C) Sequence alignment of partial predicted
502 chitin binding domain of SaChiA4 with other GH18 chitinases. The listed sequences
503 include the chitinases from *Streptomyces olivaceoviridis* (AJ276990.1),
504 *Micromonospora lupini* Lupac 08 (ZP_10087698.1), *Bacillus ehimensis*
505 (AB110080.2), *Doohwaniella chitinasigens* (AAF21468.1) and *Streptomyces griseus*
506 (2D49_A). Red triangles indicate conserved amino acid residues (Trp and Tyr).

507

508 **Fig. 2.** SDS-PAGE analysis of purified SaChiA4 from *Streptomyces albolongus*
509 ATCC 27414 expressed in *E. coli*. Lane 1, molecular mass marker of proteins; lane 2,
510 crude enzyme; lane 3, purified SaChiA4.

511

512 **Fig. 3.** Effect of pH and temperature on activity and stability of purified SaChiA4. (A)
513 The optimum pH of SaChiA4: (■) citrate buffer (pH 3.0-6.0); (●) phosphate buffer
514 (pH 6.0-8.0); (▲) Tris-HCl buffer (pH 7.0-9.0); (◆) glycine-NaOH buffer (pH
515 9.0-10.0). (B) The pH stability of SaChiA4: (■) pH 3.0; (●) pH 4.0; (▲) pH 5.0; (◆)
516 pH 6.0; (■) pH 7.0; (●) pH 8.0; (▲) pH 9.0. (citrate buffer (pH 3.0-6.0)); phosphate
517 buffer (pH 7.0); Tris-HCl buffer (pH 8.0-9.0)). (C) The optimum temperature of
518 SaChiA4. (D) The thermostability of SaChiA4: (■) 35 °C; (●) 40 °C; (▲) 45 °C; (◆)
519 50 °C; (■) 55 °C; (●) 60 °C.

520

521 **Fig. 4.** HPLC analysis of the hydrolysis products of colloidal chitin and N-acetyl
522 chitooligosaccharides (NCOSs) by purified SaChiA4. (A, B) Colloidal chitin was
523 incubated with SaChiA4 for a different time (0 min, 2 min, 15 min, 30 min, 60 min
524 and 24 h). (C-G) NCOS (DP 2-6) were incubated with purified SaChiA4 for different
525 time intervals. Standards: (N1) $(GlcNAc)_1$; (N2) $(GlcNAc)_2$; (N3) $(GlcNAc)_3$; (N4)
526 $(GlcNAc)_4$; (N5) $(GlcNAc)_5$; (N6) $(GlcNAc)_6$. (H) The calculated products of initial
527 course-time degradation (0, 2, 4, 6, 10 and 15 min) of $(GlcNAc)_6$ by SaChiA4.
528 (◆)$(GlcNAc)_6$; (▼) $(GlcNAc)_4$; (▲) $(GlcNAc)_3$; (●)$(GlcNAc)_2$; (■) GlcNAc.

529

530

Table 1
Effects of various chemical agents on SaChiA4 Activity.

Chemicals	Relative activity (%)[a]	
	1 mM	10 mM
control	100.0 ± 0.1	100.0 ± 0.1
Fe^{3+}	87.5 ± 4.9	42.7 ± 1.9
Ca^{2+}	108.2 ± 0.1	105.1 ± 6.6
Cu^{2+}	77.5 ± 0.5	37.3 ± 1.1
Mg^{2+}	123.9 ± 0.1	91.8 ± 2.6
Zn^{2+}	98.9 ± 3.7	91.5 ± 4.0
Mn^{2+}	122.7 ± 0.1	125.4 ± 0.2
Ni^{2+}	93.2 ± 2.1	91.4 ± 0.4
Ba^{2+}	120.8 ± 3.6	107.9 ± 1.8
Co^{2+}	109.5 ± 1.8	81.70 ± 3.3
K^{+}	90.7 ± 0.3	97.2 ± 0.6
Na^{+}	119.8 ± 8.6	100.1 ± 0.2
Na_2EDTA	97.3 ± 0.1	77.4 ± 0.5
SDS	35.3 ± 0.6	22.9 ± 0.2

[a] The relative activity was expressed as the percentage ratio of activity of SaChiA4 treated by various chemicals to the control without addition of reagents.

531

532

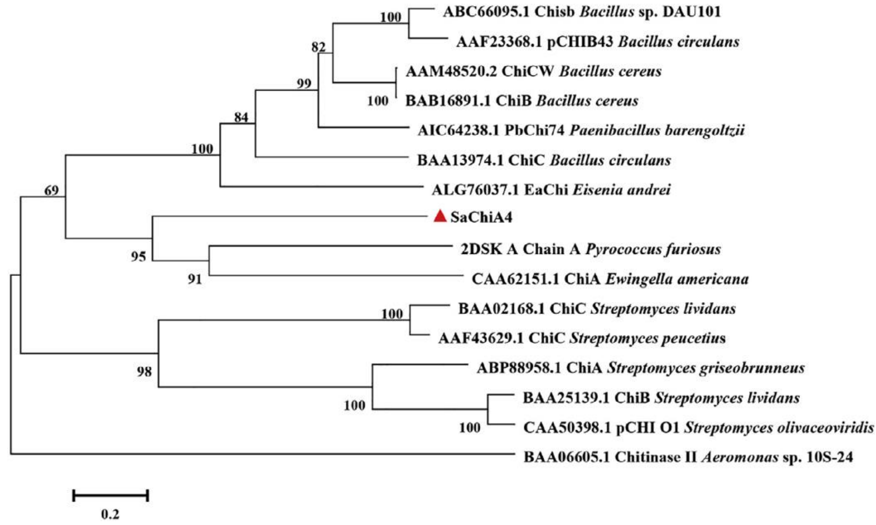
Table 2
Effects of various substrates on SaChiA4 activity.

Substrate	Relative activity (%)[b]
Colloidal chitin	100.0 ± 0.1
Chitin powder	27.5 ± 0.9
Chitosan	33.9 ± 1.2
Sodium carboxymethyl cellulose (CMC-Na)	0.8 ± 0.5

[b] The relative activity was expressed as the percentage ratio of activity of SaChiA4 using various substrates to that using colloidal chitin.

533

534



B

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SaChiA4      138  LGQTCGTPEATAAAYQKVVTKYGLKAIDFDLEEPE  172
BAA06605.1   111  MWKPAVPAPRWPRWWMPCCNAPACVPLDFDIEGSQ  145
2DSK_A       187  LCQQASTPEQLAEWYIKVIDTYNATYLDFDIEAG.  221
CAA62151.1    96  ISTKFT.VDQLVQTYTDVVQKFKAKQLDFDLENGQ  130
ABC66095.1   177  VAADPAARENFAASAVDFLRKYGFDGVDLDWEYPV  211
ALG76037.1   112  MLSSSGTRQQFIDSAISYLPRWGFDGLDLDFEYPG  146
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C

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SaChiA4          380  SWSATTTYATAGTKVSWKGHYWTNKWWTLNEDPT  413
AJ276990.1       735  AWTATKEY.TGGSVVSHKGHTFLARWWTKGEEPG  768
ZP_10087698.1   1619  AWTLTRVY.NAGDRVSFKGHVYEAKWWTRGQEPG 1652
AB110080.2        58  AWDSTKVY.TGGQKVSYNGKVFEAKWWTQGETPS   90
AAF21468.1       131  AWVSS.AY.TGGQKVTYAGRNYEAKWWTQGNVPS  164
2D49_A             8  AWSSSSVY.TNGGTVSYNGRNYTAKWWTQNERPG   41
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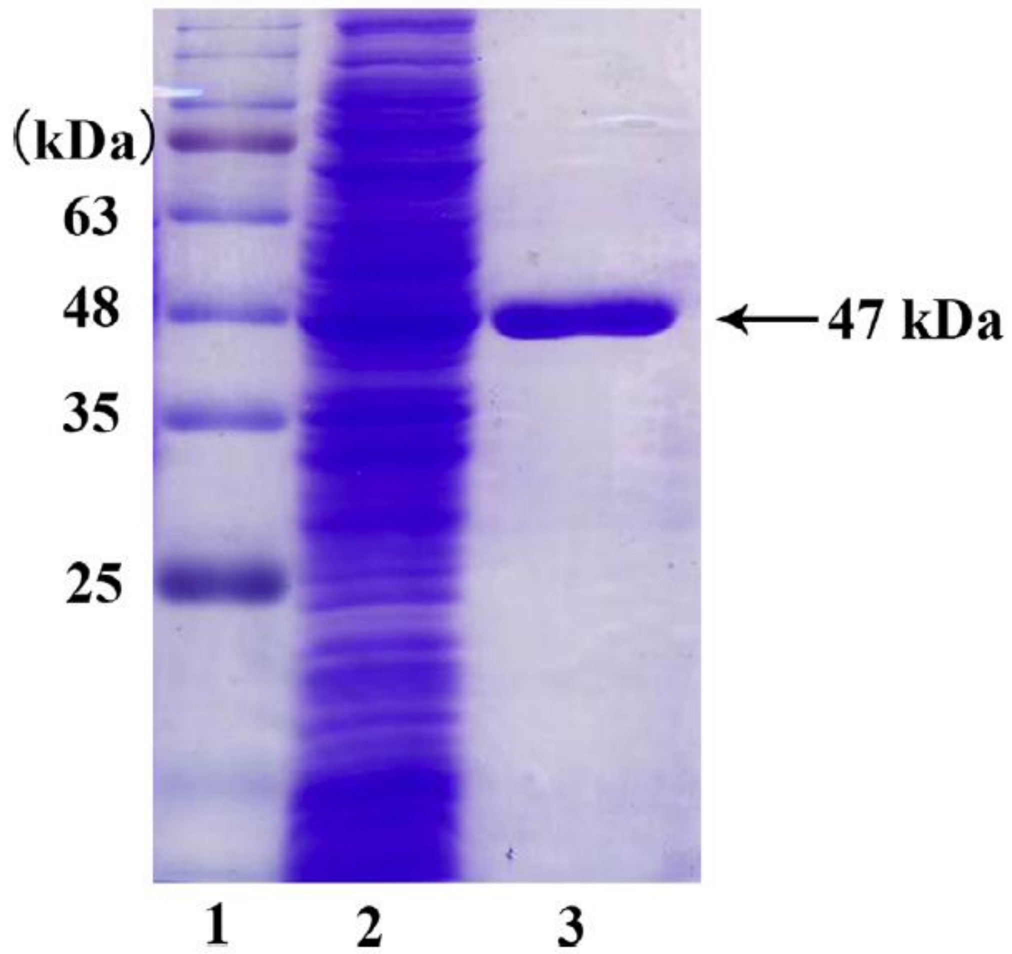
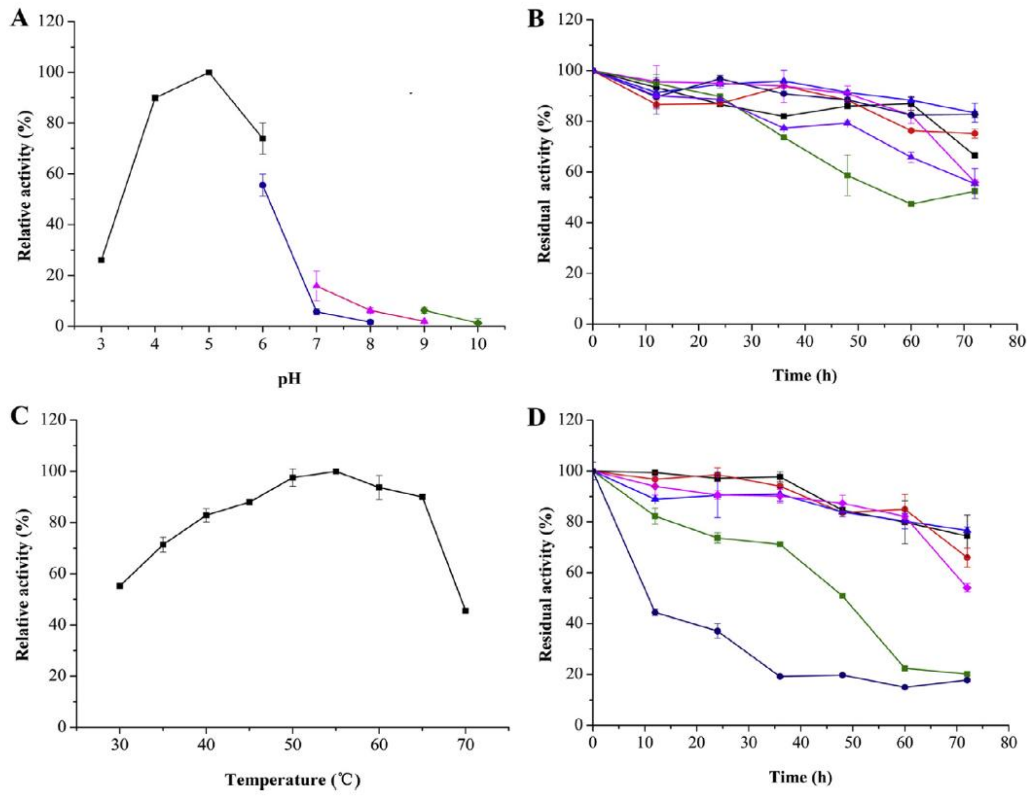
Figure 1,

538



539 Figure 2.

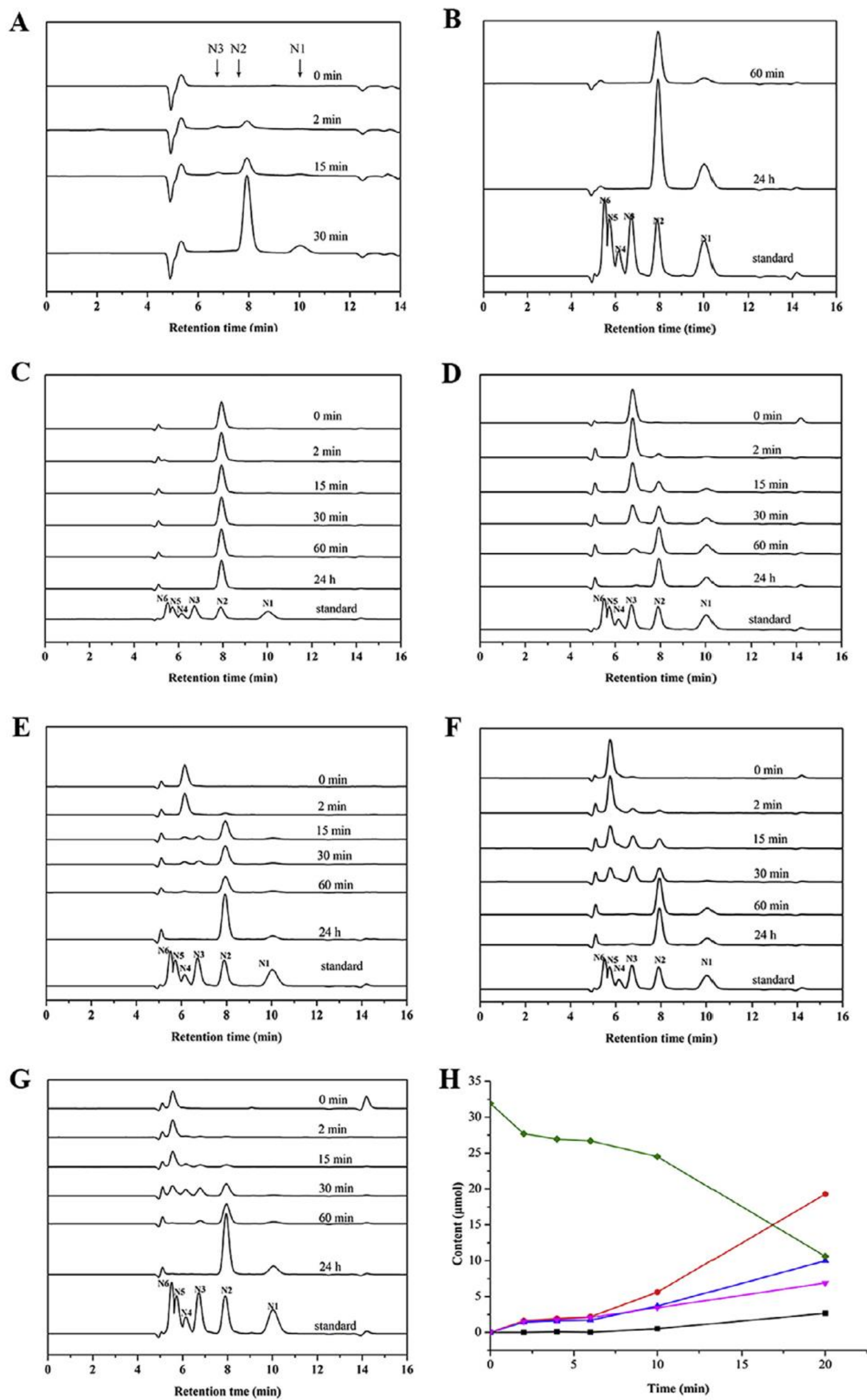
540



541 Figure 3.

542

543



544

545 Figure 4.