Cloning, Purification, and Characterization of a Novel Metagenome-Sourced Microbial Chitinase with Dual Catalytic Domains

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Research

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Cloning, purification, and characterization of a novel metagenome-sourced microbial chitinase with dual catalytic domains

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Abstract

Background: Microbial chitinases have attracted a lot of attention because of their great potential in many applications. Metagenome-based approach obtains the target genes from the environment directly without culturing the microbes and is becoming a powerful tool to discover the novel chitinases.

Results: A metagenomic approach was used to discover the chitinases from a wetland on the Qinghai-Tibetan plateau. Gene *P1724* was found and predicted to have two GH18 catalytic domains. Gene sequence containing *P1724*, only its N-terminal GH18 domain (*P1724nGH18*) or only C-terminal GH18 domain (*P1724cGH18*) were cloned and expressed in *Escherichia coli* BL21 (DE3). These purified recombinant chitinases showed maximum hydrolytic activities at 40 °C, pH 5.0-6.0 and 0-0.5 M NaCl, and were cold adaptive since they were still active at 4 °C. The activities of three chitinases were decreased with the presence of Cu^{2+} and EDTA, but increased with Ba^{2+} and Ca^{3+}. All three chitinases showed both chitotiosidase and endochitinase activities, and produced predominantly N, N′-Diacetylchitobiose from colloid chitin. Other than these common characteristics, P1724 and P1724nGH18 shared more similarity in temperature and pH stabilities, NaCl tolerance and substrate affinity, suggesting the N-terminal GH18 domain contributed more than the C-terminal GH18 did in biochemical characteristics of P1724. k_{cat}/K_m value (catalytic efficiency) of P1724 was significantly higher than the sum values of P1724nGH18 and P1724cGH18's, which indicated that two GH18 domains of P1724 works synergistically in degrading chitin.

Conclusion: Compared to the most of microbial chitinases containing only one catalytic domain, chitinases P1724 with two GH18 catalytic domains was discovered firstly by the metagenomic approach. P1724 is a novel chitinase with unique amino acid sequences and hydrolytic mode, and could be used in cold environments or industries.

Keywords: chitinases, dual GH18 domains, metagenomic approach, cold-adapted, synergy.

Background

Chitin is the second most abundant natural polymer, behind only cellulose, and a major component of fungal cell walls, the exoskeleton and gut lining of insects, and the shells of crustaceans [1]. Enzymes are biological catalysts that could speed up biochemical reactions with specificity, speed, and efficiency [2], and chitinases are ones of these enzymes and catalyze the degradation of chitin . Chitinases have shown the great application potential in the biocontrol of plant pathogenic fungi, synthesis of chitooligosaccharides for the food and pharmaceutical industry, treatment of marine wastes and production of biofuels *etc.* [1, 3]; therefore, many studies have tried to discover novel chitinases with better properties [4, 5].

So far, the most routine approach for obtaining microbial chitinases is culturing the chitinolytic microorganisms first, and then obtaining their (heterologously) expressed chitinases, such as the chitinases: *Ss*Chi18A, *Ss*Chi18B, and *Ss*Chi18C from *Streptomyces* sp. F-3 [6], *Cm*Chi1 from *Chitinolyticbacter meiyuanensis* SYBC-H1 [7], MtCh509 from *Microbulbifer thermotolerans* DAU221 [8] , PbChi74 from *Paenibacillus barengoltzii* [9], *etc*. However, it is well known that the vast majority (up to 99–99.9%) of microorganisms present in nature, especially those in the extreme environments, cannot be cultured under laboratory conditions, which is one of the biggest obstacles to discover the novel enzymes/chitinases [10]. Metagenome-based approaches are the complete access to the entire community's genetic pool without the need of microbial cultivation [11], which have been used to discover a few chitinases [12]. These metagenome-sourced chitinases could be used for the biocontrol of pathogenetic fungi [13] and insects [12, 14], an additive in the food and feed industries

[15], as well as industrial processing of chitin [16].

Based on amino acid sequence similarity, chitinases are grouped into glycosyl hydrolase (GH) families 18, 19, and 20 [1]. Bacterial chitinases mainly belong to GH18, some *Streptomyces* chitinases are GH19, and GH20 includes the β-N-acetylhexosaminidases from bacteria [1]. GH18 chitinases usually contain one catalytic GH18 domain and several auxiliary domains [5], while four microbial chitinases with dual GH18 catalytic domains have been reported so far [17-20]. These chitinases with dual GH18 domains had shown the advantages over the ones with a single catalytic domain due to: 1) the two GH18 domains in combination exhibited a significantly higher activity than the sum of their individual activities and/or 2) the two domains contributed different catalytic modes [15]. For examples, in the chitinase *Tk*-ChiA from *T. kodakaraensis* KOD1 and the chitinase B from "*Microbulbifer degradans*" 2-40 (now *Saccharophagus degradans* 2-40 [21]), the N-terminal and C-terminal GH18 domains functioned as an exo-chitinase and an endo-chitinase respectively [18, 19].

Here we reported the first metagenome-sourced chitinase that contained two GH18 catalytic domains. In our previous study, we found the microbial communities from the Qinghai–Tibetan Plateau wetland soils could effectively convert chitin to methane at low temperatures [22]. We continued to anaerobically enrich the microbial community from Haiyan wetland soil with chitin flakes as a sole carbon resource, and then next-generation metagenomic sequencing was done on the community. Gene *P1724* predicted to have two GH18 catalytic domains was discovered, and then cloned, expressed, purified, and characterized. In addition, two GH18 domains of P1724 were individually cloned, expressed, purified, and characterized in *E. coli*. The results showed the P1724 is a novel chitinase with two GH18 domains working synergistically and could be used in cold environments or industries.

Results

Discovery and sequence analysis of P1724

A chitin-enriched microbial community was obtained from the Haiyan wetland soil on the Qinghai-Tibetan plateau after two-years of repeated subculturing. Next-generation metagenomic sequencing and CAZyme annotation revealed that the metagenome of the chitin-enriched microbial community encoded a broad array of potential enzymes (details were shown here). Gene *P1724* (2880 bp) was found and encoded a protein (960 amino acids) containing GH18 domains (the details of the sequences can be seen in the additional file). Based on the InterPro search, P1724 was composed of four domains, two GH18 domains and two carbohydrate-binding modules of family 5/12 (CBM 5/12) (Fig.1). Most likely P1724 does not contain secretion signal since a sec signal peptide was predicted by using the SignalP-5.0 server with only 0.4283 of the likelihood. However, the InterPro search showed the P1724 was in the extracellular region, which corresponded to the fact that chitinases were often secreted enzymes. The amino acid sequence of P1724 was compared with the sequences in non-redundant protein sequences database (nr) in GenBank, and had the best BLAST hit (95% query cover and 60.10 % identity) with a hypothetical protein from *Clostridium* sp. Marseille-P299 (WP_082812200.1), and also showed 36.2 % identity (96 % query cover) to a hypothetical protein from *Jiangella alkaliphila* (WP_082155267.1) and 33.8 % identity (97 % query cover) to a chitinase from *Paenibacillus* sp. UNC499MF (WP_103997363.1). These proteins retrieved from the GenBank may be also made up of two distinct catalytic domains, but have not been further studied. Based on the unique primary structure and similarity analysis, we concluded that P1724 was a novel chitinase.

The two catalytic domains of P1724 located from residues 28 to 397 and 669 to 941 of

P1724 respectively (Fig. 1). BLAST analysis revealed that the amino acid sequence of the N-terminal and C-terminal GH18 domains (nGH18 and cGH18) were only 24.4 % identical and phylogenetically distanced to each other (Fig. 2). Figure 2 also showed10 sequences that had the highest identities (79.9- 49.4%) with nGH18 or cGH18 in nr database; these sequences have not been characterized so far and belonged to *Clostridium* sp. Marseille-P299, *Lachnoclostridium* spp., *Anaerocolumna* spp., *Ruminiclostridium* spp. *etc.* For the nGH18, the InterPro search also showed it contained a Chitinases Insertion Domain (CID) from residues 292 to 368, while cGH18 had no such domain (Fig.1). The BLAST search in UniProtKB/Swiss-Prot database showed that nGH18 had the best hit (43.1%) with the domain of Chitinase A1 of *Bacillus circulans*, while cGH18 had the best hit (35.90%) with Chitinase D of *B. circulans.* Chitinase A1 and Chitinase D belong to chitinase subfamily A and B respectively [23], and so nGH18 and cG18 should belong to different subfamilies as well.

Cloning, expression, and purification of recombinant P1724 and its individual GH18 domain

Three fragments (Fig.1), *P1724* (nt 31-2880), *P1724nGH18* containing nGH18 (nt 31 to 1884) and *P1724cGH18* containing cGH18 (nt 1372 to 2880) were amplified by PCR and then cloned into the pCold I respectively. The first 10 amino acids were eliminated to avoid their potential effect on the solubility of recombinant protein. All fragments with an N-terminal $(His)_6$-tag were heterogeneously expressed in *E.coli* BL21 (DE3). The constructions of *P1724nGH18* and *P1724cGH18* were made to investigate how these two GH18 domains function individually. If the position of CMBs, at the middle, N-terminal or C-terminal position of proteins, would not affect the catalytic abilities, (Fig. 1), the only difference between the three recombinant proteins would only rely on

their catalytic domain(s). Figure 3a showed the molecular weight analysis of three purified recombinant proteins, and their molecule weight mostly corresponded to their theoretical molecular mass 102.9, 67.5, and 53.9 kDa calculated by ProtParam Tool [24]. The Native PAGE and zymogram showed there were clear zones around three purified protein bands (Fig. 3b, 3c).

Effects of temperature on the activities of P1724 and its individual GH18 domains

To estimate their optimum hydrolytic temperatures, all three purified recombinant chitinases were incubated at 4-60 °C for 1 h using colloid chitin as the substrate. The results showed that P1724, P1724nGH18, and P1724cGH18 achieved maximum activities around 40 °C (Fig. 4a). The maximum activities were set to 100% then. Fig. 4a showed that three chitinases were still active at low temperatures, for example, three chitinases kept 16-35% of their maximal activities at 4 °C; at 45-50°C, P1724cGH18 still kept 100% of its maximal activity, while the activities of P1724 and P1724nGH18 dropped significantly. For the estimation of temperature stabilities, the recombinant chitinases were preincubated at 4-60 °C without the substrate for an hour, and then assayed for their residual hydrolytic activities at 40 °C. The activities of three chitinases preincubated at 4 °C were set to 100%. Figure 4b showed all three chitinases were stable at 4–40 °C and kept ~100% activities. Preincubated at 45 °C, P1724 and P1724nGH18 almost lost all of their hydrolytic activities, while P1724cGH18 still kept 100% of its. Overall, compared to P1724 and P1724nGH18, P1724cGH18 was more active and stable at higher temperatures.

Effects of pH on the activities of P1724 and its individual GH18 domains

The effects of pHs on the recombinant chitinases were determined at pH 3.0-10.0 buffer systems. Chitinase P1724, P1724nGH18, and P1724cGH18 exhibited maximum activities around pH 5.0-6.0 (Fig. 4c). The maximum activities of three chitinases were

191 set to 100%. Figure 4c showed that their activities dropped significantly (> 50%) when
192 at the pH ≤ 3.0 and pH ≥ 7.0. For estimating the pH stabilities, the recombinant
193 chitinases were kept in pH 3.0-10.0 buffers at 4 °C for 24 h, and then assayed for their
194 residual hydrolytic activities at pH 6 and 40 °C. All three chitinases were stable and
195 showed the highest activities after preincubated for 24 h at pH 5.0 and 6.0, and then
196 these highest activities were set to 100% (Fig. 4d). When at neutral and alkaline
197 conditions (pH 7.0-10.0), all recombinant chitinases could keep relatively high activities
198 (> 50%) (Fig. 4d). When preincubated at pH 3.0 and 4.0, P1724 and P1724nCH18 lost
199 more than 80% of their maximum activities, while P1724nCH18 was not affected at all
200 and kept 100% of its maximum activity (Fig. 4d). Overall, compared to P1724 and
201 P1724nGH18, P1724cGH18 could adapt wider pH range.
202 **Effects of NaCl and metal ions on the activities of P1724 and its individual GH18**
203 **domains**
204 The effects of NaCl on the recombinant chitinases were tested in the reaction mixtures
205 with the final concentrations of 0-3 M of NaCl. The activities of three chitinases were
206 set to 100% at 0 M NaCl. All three chitinases kept about 100% activities at 0.5 M NaCl,
207 but decreased linearly at higher concentrations of NaCl (Fig. 5). P1724 and
208 P1724nGH18 lost 50% their activities around 1.25 M NaCl, while P1724cGH18 lost
209 about 50% when around 1.75 M NaCl. The effects of metal irons were estimated in the
210 reaction mixtures with 1 mM or 5 mM of the metal irons. These irons were Ba^{2+}, Ca^{2+},
211 Co^{2+}, Cu^{2+}, Fe^{3+}, K^{+}, Li^{+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, as well as EDTA and dithiothreitol (DTT).
212 The activities of three recombinant chitinases were set to 100% at the concentration of 0
213 M of the compounds. For P1724, its activities were increased (> 100%) when most of
214 the irons presented in the reaction, but were obviously decreased (< 100%) when Cu^{2+},
215 Fe^{3+}, and EDTA presented (Fig. 6a). The activities of 1724nGH18 decreased with the

presence of $Cu^{2+,}$ Co^{2+}, Fe^{3+}, Mg^{2+}, and EDTA, but increased with Ba^{2+} and Ca^{3+} (Fig. 6b). For P1724cGH19, its activities obviously increased with the presence of Ba^{2+}, Ca^{2+}, Fe^{3+}, Li^{+}, and DTT, and decreased when Cu^{2+}, Co^{2+}, and EDTA presented (Fig. 6c). In general, Cu^{2+} and EDTA had negative effects on the P1724 and its individual domains, and Ba^{2+} and Ca^{3+} had positive effects on them; Fe^{3+} had negative effects on P1724 and its nGH18 domain, but showed a positive effect on the cGH18 domain.

Substrate specificity of P1724 and its individual GH18 domains

Other than colloid chitin, the recombinant chitinases were also tested to see if they could hydrolyze on other polysaccharides as indicated in Table 1. When the colloid chitin was used as the substrate, P1724 had the highest specific activity, followed by P1724nGH18, and then P1724cGH18; the specific activity of P1724 is about 0.3 U/mg higher than the sum of P1724nGH18 and P1724cGH18 (Table 1). All three chitinases showed much higher hydrolytic activities towards the fluorimetric 4-Methylumbelliferyl (MU)-β-D-N, N´-diacetylchitobioside hydrate and 4-MU-β-D-N, N´, N´´ -triacetylchitotriose than other tested polysaccharides, and no obvious hydrolytic activity on 4-MU N-acetyl- β -D-glucosaminide (Table 1); in another word, all three chitinases had chitobiosidase and endochitinase activities, and no β-N-acetylglucosaminidase activity; In addition, all three chitinases showed different advantages in the hydrolytic mode; P1724 and P1724nGH18 had a higher endochitinase activity, while P1724cGH18 showed higher chitobiosidase activities. All three chitinases also showed the hydrolytic activities towards 10% chitin powder at 4 °C and 40°C. All three chitinases could hydrolyze the colloid chitosan, but not the powdery chitosan. No activities were detected for 1% chitin powder, CMC, and microcrystalline cellulose (Table 1).

Table 1. Specific activities of three recombinant chitinases toward various substrates

Substrates	P1724 (U/mg)	P1724nGH18 (U/mg)	P1724cGH18 (U/mg)
colloid chitin	2.9±0.2	1.5±0.1	1.1±0.1
4-MU N-acetyl-β-D-glucosaminide	ND*	ND	ND
4-MU-β-D-N, N´-diacetylchitobioside	1076.2±8.0	396.4±20.2	729.2±75.5
4-MU-β-D-N, N´, N´´-triacetylchitotriose	1198.2±26.2	783.5±38.3	225.2±19.7
Chitin powder (10%) at 40°C /4°C	0.15/0.03	0.06/0.02	0.03/0.02
Chitin powder (1%)	ND	ND	ND
Colloid chitosan (DS≥90%)	0.09	0.09	0.06
Chitosan powder (DS≥90%)	ND	ND	ND
CMC	ND	ND	ND
Microcrystalline cellulose	ND	ND	ND

*, not detected.

Kinetic parameters

Using the colloid chitin as the substrate, the kinetic constants of the three chitinases were determined and shown in Table 2. P1724 had the lowest K_m value, followed by P1724nGH18, and then P1724cGH18; K_m values of P1724 and P1724nGH18 were close and lower than 1 mg /mL, but the value of P1724cGH18 was much higher and about 2.1 mg /mL. Generally, an enzyme with a low K_m has a high affinity for its substrate; therefore, P1724 and P1724nGH18 had the higher affinity for the colloid chitin than P1724cGH18. P1724 also had the highest k_{cat} and K_m /k_{cat} values (Table 2), and so the catalytic efficiency; K_m /k_{cat} values of P1724 was obviously higher than the sum values of nGH18 and cGH18 working individual, suggested that the individual domains of P1724 working cooperatively to achieve higher hydrolytic efficiency.

254 Table 2. Kinetic parameters of three recombinant chitinases toward colloid chitin.

Parameters	P1724	P1724nGH18	P1724cGH18
K_m (mg/mL)	0.6±0.1	0.8±0.1	2.1±0.3
V_{max}	3.2±0.1	1.8±0.12	1.7±0.1
k_{cat} (s^{-1})	5.4	2.0	1.5
k_{cat}/K_m (mL/s· mg)	9.0	2.5	0.7

255

256 **Hydrolysis pattern of the P1724 and its individual chitinolytic domains**

257 The hydrolysates of the recombinant chitinases towards colloid chitin were analyzed by
258 the thin layer chromatography (TLC) method. After the reaction mixtures were
259 incubated at 40 C for 1 h, 1 ul of each reaction mixture or 1ul of each 15-time
260 concentrated reaction mixture was loaded on the TLC plates. Without being
261 concentrated, only N, N´-diacetylchitobiose $(GlcNAc)_2$ was detected on the TLC plate
262 from all three chitinases (Fig. 7a). After being concentered, $(GlcNAc)_2$ was the densest
263 product on the plate, and GlcNAc and other oligomers $(GlcNAc)_{>2}$ also showed up on
264 the plate (Fig. 7b).

265 ## Discussion

266 In this study, we cloned, expressed, and characterized a chitinase (P1724) from the
267 metagenome of a chitin-enriched community. Different from most of the microbial
268 chitinases that contain only one catalytic domain, P1724 contains two. So far, four
269 microbial chitinases with dual GH18 domain had been characterized, and they are
270 Tk-ChiA from Archaeon *T. kodakaraensis* KOD1 [18], Chitinase B from *S. degradans*
271 2-40 [19], VChti-1 from Chlorella Virus [17], and ChiW from *Paenibacillus* sp. strain
272 FPU-7[20, 25]. These chitinases were all obtained from the purified microorganisms,
273 and so P1724 is the first microbial chitinase with dual catalytic domains discovered by
274 the metagenomic approach.

Chitinase P1724 was obtained from a community incubated under a cold and anoxic condition. The amino acid sequence of P1724, its nGH18 or cGH18 showed the highest identities with the hypothetical proteins or chitinases from anaerobes, such as *Clostridium* sp. Marseille-P299, *Lachnoclostridium* spp., *Anaerocolumna* spp., *Ruminiclostridium* (Fig. 2); therefore, P1724 should belong to an anaerobe as well. So far, only few studies focused on chitin degradation in cold-area wetlands under anoxic conditions although anaerobic fermentation of chitin could be one of the important processes of nitrogen cycling in such environments [22, 26, 27]. Using the next-generation metagenomic method, we found this novel chitinases P1724 and avoided culturing the cold-favoring chitinolytic anaerobes, such as *Paludicola psychrotolerans*, which were normally hard to handle and very slow-growing [28]. Once again, our study supports that the metagenomic method is a powerful tool to discover new enzymes from extreme environments [29, 30].

Chitinase P1724 showed cold-adapted capacity. So far, most of the chitinases that had been characterized exhibited the highest activity ≥ 50 °C, and a few chitinases had been reported to be psychrotolerant or psychrophilic with optimal working temperature ≤ 30 °C [31, 32]. Although P1724 showed the highest hydrolytic activities at 40 °C, which is higher than most of the psychrotolerant chitinases reported, P1724 is still cold-adapted since it could hydrolyze colloid and powdery chitin at the low temperatures, such as 4 °C (Table 1). So far, cold-adapted or psychrophilic chitinases have shown the advantages in some applications where the low temperatures were demanded or could be not avoid, such as the preparation of chitooligosaccharides used for food or drugs, and the biocontrol of plant pathogens in the field and spoilage microorganisms in refrigerated food [31]. Therefore, P1724 could have the potential to be applied in such industries.

Two GH18 domains of P1724 are distinctive to each other in sequences and have unique hydrolytic modes. Based on the amino acid sequence similarity, GH18 chitinases could be classified into three subfamilies A, B, and C [33]. The main structural difference among these subfamilies is that a chitinases insertion domain (CID, a small α + β domain) inserts into the TIM barrel catalytic domain in the subfamily A, while subfamilies B and C have no such domain [33]. nGH18 of P1724 contains a CID, while cGH18 does not (Fig. 1); therefore, nGH18 should belong to subfamily A, while cGH18 should belong to subfamily B since it has best sequence similarity with subfamily B -type Chitinase D from *Bacillus circulans* [23]. Generally subfamily A chitinases are believed to be processive exochitinases, while subfamily B chitinases are non-processive modular endochitinases containing a catalytic domain and one or more CBMs [34]. Although P1724cGH18 made in this study matches the subfamily B chitinase module (one catalytic domain and two CBMs), it showed both (relatively weaker) endochitinase and (stranger) exochitinase activities (Table 1). P1724nGH18 containing one subfamily A catalytic domain and two CBMs showed not only the exochitinase activity like other subfamily A chitinases [34], but also the endochitinase activity (Table 1). Certainly, P1724, which is made up of nGH18 and cGH18, also showed both endochitinase and exochitinase activities. Further studies on crystal structure of chitinase-substrate complexes may help to elucidate the unique hydrolytic modes of P1724 and its nGH18 and cGH18 domains. Chitinase that have two or three different catalytic modes among endochitinase, exochitinase and N-acetylglucosaminidase is considered to have broad specificity [35]. Compared with the chitinases with single catalytic mode, chitinases with broad specificity have more advantages in lowering the cost in the extraction process and increasing the catalytic efficiencies of the enzymes [35]. Therefore, we believe P1724, P1724nGH18 and

P1724cGH18 could have advantages in applications due to their broad specificity. P1724 shared more similar in biochemical features with its nGH18 domain than its cGH18 domain. In responding to the temperatures, unlike cGH18 domain (P1724cGH18) was still stable around 45°C, P1724 and its nGH18 domain (P1724nGH18) lost most of the activities at 45°C or preincubated at 45°C significantly (Figure 4a,4b). In responding to the pHs, unlike cGH18 was stable through pH 3-10, P1724 and nGH18 domain lost most of their activities at pH 3.0 and 4.0 (Figure 4d). In term of tolerances to the salt, P1724 and nGH18 lost 50% activities around 1.25 M NaCl, while cGH18 was more tolerant and lost 50% of its maximus activity at higher concentration of NaCl (~ 1.75 M). In responds to metal irons, Fe^{3+} decreased the activities of P1724 and nGH18, but had a contrary effect on cGH18. In addition, P1724 and nGH18 domain had the similar the K_m values, and so the similar affinities to the colloid chitin, while the K_m values of cGH18 was much higher than theirs (Table 2). Therefore, we would conclude nGH18 domain contributed more than cGH18 did in the biochemical features of P1724.

P1724 showed synergy between its two GH18 domains in term of catalytic efficiency. P1724 had highest catalytic efficiency, and its k_{cat}/K_m value was significantly higher than the sum value of nGH18 and cGH18. Studies already discussed that the chitinases with two catalytic domains had the advantages over the ones with a single catalytic domain [15]. Among many chitinases with a single catalytic domain that had been studied for their kinetic parameter for colloid chitin [1, 36-38], chitinase A from *Vibrio harveyi* [39] and EcChi1 from *Enterobacter cloacae* subsp.cloacae [40] had a similar optimum temperature (37-40°C) with P1724. Chitinase A had K_m, k_{cat} and k_{cat}/K_m values of 12 ±1.4 mg/mL, 0.10 s^{-1} and 83×10^{-4} mL/s·mg respectively, and EcChi1 had K_m, k_{cat}, and k_{cat}/K_m values of 15.2 mg/mL, 0.16×10^{2} min^{-1} and 0.011×102 mL/s·mg

respectively. Compared with these two chitinases, P1724 including its individual catalytic domains indeed had higher affinities and catalytic efficiencies towards the colloid chitin since they showed lower K_m and higher k_{cat}, and k_{cat}/K_m values. Therefore P1724 might have a better application potential.

P1724 and its individual catalytic domains showed better hydrolytic activities towards amorphous form than solid form of the substrates. Other than colloid chitin and soluble diacetylchitobiosid, triacetylchitotriose, and also glycol chitin in the zymogram test, P1724 and its individual catalytic domains also showed hydrolytic activities towards 10% powdery chitin, but not 1% powdery chitin, which suggested that they had lower affinity toward the crystalline form of chitin than the colloidal form. As for chitosan, P1724 and its individual catalytic domains could hydrolyze colloid chitosan, but not 1% or 10% powdery chitosan, further confirmed that P1724 and its individual catalytic domains had better hydrolytic activities towards amorphous form than solid form of substrates. This is most likely due to colloidal chitin or chitosan are more accessible for the active site of chitinases than their crystalline form [1]. A few other chitinases had been reported to have hydrolytic activities on chitosan, such as Chitinase Chi1 from *Myceliophthora thermophila* C1 [41], a chitinase from *P. timonensis* strain LK-DZ15 [37] and Chit42 from *Trichoderma harzianum* [42]. Previous studies reporting that the GH18chitinases were able to cleave the glycosidic linkage of not only GlcNAc-GlcNAc in chitin but also GlcNAc-GlcN present in chitosan [43], which would explain that P1724 and its individual GH18 domains could hydrolyze chitosan as well.

P1724 and its catalytic domains showed various sizes of hydrolytic products towards colloid chitin, and $(GlcNAc)_2$ was the major product. As discussed previously, all three chitinases showed both chitobiosidase and endochitinase activities and no β-N-acetylglucosaminidase activity. Endochitinase activity of all three chitinases could

375 produce wide range of oligomers $(GlcNAc)_{\geq 2}$, and chitobiosidase activity mainly
376 produced $(GlcNAc)_2$. Endochitinase and chitobiosidase both produce $(GlcNAc)_2$, which
377 should not be further hydrolyzed to GlcNAc without β-N-acetylglucosaminidase,
378 therefore, $(GlcNAc)_2$ became the predominant products. In addition, oligomers
379 $(GlcNAc)_3$ could be hydrolyzed further by chitobiosidase to $(GlcNAc)_2$ and GlcNAc,
380 and so GlcNAc showed up as one of final products. Chitin oligosaccharides have
381 attracted much attention in recent years because of their functions as antimicrobial,
382 anti-tumor, hypoglycemic agents and plant elicitors [44]. P1724, P1724nGH18 and
383 P1724cGH18 obtained in this study could be useful chitinase resources to produce
384 chitin oligosaccharides from chitin biomass in a greener industrious process compared
385 to the traditional processes using concentrated acids or alkalis [8].

386 **Conclusion**

387 In summary, we discovered, obtained and characterized a chitinase with dual GH18
388 catalytic domains from a wetland on the Qinghai-Tibetan plateau using the
389 metagenomic and genetic engineering approaches. The results showed that the chitinase
390 (P1724) is unique in the amino acid sequence and catalytic mode. One of catalytic
391 domains in P1724 played a dominant role in the biochemical characteristics of P1724,
392 while both domains worked synergistically and made P1724 having higher catalytic
393 efficiency. P1724 and its individual domains have broad specificity and are
394 cold-adapted, and so could have an application potential in the industries or
395 environments requiring low temperatures.

396 **Methods**

397 **Microbial consortium enrichment**

398 A soil sample was collected from Haiyan wetland on the Qinghai-Tibetan Plateau,
399 China (101°69'N, 36°.58'E, 2,986 m above sea level) in July 2013, and then was

processed and incubated with chitin flakes as a sole carbon resource at 15 °C under anaerobic condition. After subcultured repeatedly for two years, the microbiome was collected by centrifugation and used for next-generation metagenomic sequencing. For more detailed information on sample processing, please refer to our previous publication [22].

Metagenomic sequencing and CAZyme annotation

The total DNA was extracted, sonicated, and used to construct a paired-end library with an insert size of ~450 base pairs DNA. The Illumina HiSeq platform (paired-end, 2×150 bp) were used for sequencing. Abound 7.5 Gb data high-quality reads were generated and assembled using IDBA (http://i.cs.hku.hk/~alse/hkubrg/projects/idba_ud/). Gene-coding sequences were predicted on the assembled contigs using MetaGeneMark [45]. Predicted proteins were used to do CAZyme annotation in dbCAN (http://csbl.bmb.uga.edu/dbCAN/annotate.php). Gene *P1724* was discovered and predicted to have two GH18 catalytic domains.

Domain and sequences analyses

The InterProScan was used to search and analyze conserved domains and signature sequences (http://www.ebi.ac.uk/interpro/search/sequence/). The database homology search of the nucleotide and amino acid sequences was performed at NCBI BLAST [46]. The ProtParam tool was used to calculated the physicochemical characteristics (http://web.expasy.org/protparam/) [24]. The signal peptide was analyzed at the Signal P 3.0 server (http://www.cbs.dtu.dk/services/SignaIP-3.0). Phylogenetic analysis and tree of amino acid sequences was carried out in the Interactive Tree of Life online tool (http://itol.embl.de/).

Gene cloning

P1724 has 2883 base-paired nucleic acids and encodes a protein with 961 amino acids.

In case the first 10 amino acids may be the secretion signal and have an effect on the solubility of recombinant protein, they were eliminated, and then a 2853-bp fragment (nt 31 to 2880) of *P1724* was amplified by PCR from the total DNA. The PCR primers were P1724F_KpnI (5'-CCGGGGTACCCACGTATCGGCAAATGAGAACT-3') and P1724R_BamHI (5'-GCGCGGATCCCTATTCAAACTCAGCAAAAC-3'). In addition, the fragment (nt 31 to 1884) of P1724 containing N-terminal GH18 catalytic domain and the CMBs was amplified using the primer pair of P1724F_KpnI and P1724(1884)R_BamHI (5'-GCGCGGATCCCTATATTACACCCCCTATTTTG-3'), and the fragment (nt 1372 to 2880) containing C-terminal GH18 catalytic domain and the CMBs was amplified using the primer pair of P1724(1372)F_KpnI (5'-CCGGGGTACCATACCGACTCCTACACCAA T-3') and P1724R_BamHI. All obtained PCR fragments were cloned into the vector pCold I (Takara Biomedical Technology (Beijing) Co., Ltd, China). The resultant constructs were transformed into *E. coli* BL21 (DE3) cells for the overproduction of the recombinant proteins.

Gene expression and protein purification

Overnight cultures of *E. coli* strain BL21 harboring the recombinant plasmids were used to inoculate LB broth (10 g/L peptone, 5 g/L yeast extract, and 5 g/L sodium chloride, pH 7.0) containing 100 μg/mL ampicillin. The cultures were incubated at 37 ℃ and 200 rpm until an optical density of 0.4-0.5. The cultures were then quickly refrigerated at 15 ℃ and left to stand for 30 minutes. IPTG was added into the culture at the final concentration of 1.0 mM, and then the cultures were continued to shake at 15 ℃ for 24 hours. The recombinant proteins (chitinases) were extracted using Ni-NTA resin (QIAGEN China (Shanghai) Co., Ltd.) as follows: cells were harvested by centrifugation at 5,000 x g for 10 min at 4°C and then resuspended in the non-denaturalizing lysis buffer with lysozyme (1mg/mL). The cell suspensions were left

on ice for 30 min and then sonicated for 1 second with 1 second interval for 20 min (200 W). After sonification, the cell suspensions were centrifuged (10,000 g, 30 min, at 4°C) to remove the insoluble cell debris. The supernatants were immediately applied to the Ni–NTA agarose affinity column and the chromatography was carried out gravitationally at 4 °C. The columns were washed with the wash buffer (20 mM Tris–HCl buffer, pH 8.0 and 150 mM NaCl) containing 20 mM imidazole. Ni–NTA bound proteins were eluted with 250 mM imidazole in the same buffer. To remove imidazole, the eluted fractions were then dialyzed using the cellulose membrane tubes (Solarbio Science & Technology, Co., Ltd, Beijing, China). The purified proteins were stored at -80 °C until used.

Determination of protein quantification, molecular weight and zymogram

The concentrations of recombinant proteins were measured by BCA protein assay kit (Beyotime Biotechnology, Jiangsu, China). The molecular weights of the recombinant chitinases were determined using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) method. The protein size was estimated using standard protein markers (Takara Biomedical Technology (Beijing) Co., Ltd, China). After separating the samples, the gel was stained with Coomassie Blue Fast Staining Solution (Beyotime Biotechnology, Jiangsu, China). To detect chitinase activity, the zymogram method was used as indicated by Lee *et al.*[8]. Briefly, the protein samples were loaded into two native gels, one with and one without 0.1% glycol chitin. After electrophoresis, the gel without glycol chitin were stained by Coomassie Blue Fast Staining Solution, while the gel with 0.1% glycol chitin was incubated in refolding buffer [50 mM citrate buffer (pH 6.0), 1% Triton X-100] at 40 °C for 10 min. Then, the gel was stained with 0.01% calcofluor white M2R in 50 mM citrate buffer (pH 5.0), rinsed several times with distilled water and visualized on a UV transilluminator.

475 **Chitinase activity assay**

476 Chitinase activities were determined by the dinitrosalicylic acid (DNS) method of
477 Miller [47] using colloid chitin (from crab shell flakes, Biotechnology (Shanghai) Co.,
478 Ltd., China) as the substrate. Colloid chitin was made by the procedure of Li *at el*[48].
479 The standard enzyme reaction mixture was as follows: 5-10 μL purified enzyme
480 solution, 90 μL 1% colloidal chitin, 100 μL 100 mM citrate buffer (pH 6.0). The
481 standard assay condition was the reaction mixtures were incubated at 40 °C for 1 h. One
482 unit of activity was defined as the amount of enzyme catalyzing the production of 1
483 μmol of N, N′-diacetylchitobiose $(GlcNAc)_2$ per min.
484 When using fluorimetric substrates, 4- MU-acetyl-β-D-glucosaminide, 4-MU N, N′-
485 diacetylchitobioside hydrate and 4-MU β-D-N, N′, N′′- triacetylchitotriose, the assays
486 were carried out following the protocol suggested by the Chitinase Assay Kit
487 (Sigma-Aldrich Corporation, USA); 4-MU N, N′- diacetylchitobioside and
488 4-MU-acetyl-β-D-glucosaminide were used for the detection of chitobiosidase and
489 β-N-acetylglucosaminidase activities (exochitinase activity) respectively, and
490 4-MU-β-D-N, N′, N′′-triacetylchitotriose was employed for measurement of
491 endochitinase activity.

492 **Effects of pH, temperature, NaCl and metal irons on chitinase activities**

493 The optimal pH of the chitinase activity was measured between pH 3.0–10.0 in the
494 enzyme reaction mixtures with different buffers: citrate buffer (pH 3.0-6.0), phosphate
495 buffer (pH 7.0-8.0), and Glycine-NaOH buffer (pH 9.0-10.0). To determine the pH
496 stability of the chitinase activity, the recombinant chitinases were incubated at various
497 pHs using the above mentioned buffers at 4 °C for 24 h, and then the remaining enzyme
498 activity was measured in the standard enzyme reaction mixture and assay condition. The
499 optimum temperature of the chitinase activity was studied by incubating the enzyme

samples in the standard reaction mixture at the temperatures of 4-60 °C. To determine the thermal stability of the chitinases, enzyme samples in 50 mM citrate buffer (pH 6.0) was incubated at the temperatures of 4 to 60 °C for 1 hr, then the remaining enzyme activities were measured in the standard enzyme reaction mixture and assay condition. The effect of salt on the chitinases activity was measured in the presence of 0, 0.5, 1, 1.5, 2, 2.5 and 3M NaCl; the effects of metal ions and reducing agents on the chitinases activity were assayed in the presence of 1 and 5 mM of each ions, EDTA and DTT; these irons included Ba^{2+}, Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{3+}, K^{+}, Li^{+}, Mg^{2+}, Mn^{2+} and Zn^{2+}; each of these compounds was accordingly supplemented to the standard enzyme reaction mixture, which then was tested in standard assay condition.

Substrate specificities and kinetic parameters of the recombinant chitinases

Substrate specificities of the purified chitinases were assayed at 40 °C and 4 °C in 50 mM citrate buffer (pH 6.0) using various substrates. The tested substrates included 1% (w/v) for colloidal chitin, chitosan and carboxymethyl cellulose (CMC, Shanghai Macklin Biochemical Co., Ltd.(China), 1% and 10 % for chitin powder from shrimp shells (Sigma-Aldrich Corporation, USA), and 10% chitosan powder and crystalline cellulose (Biotechnology (Shanghai) Co., Ltd., China).

Kinetic parameters of the recombinant chitinases for colloidal chitin were determined. The recombinant chitinases were mixed with various concentrations of colloid chitin (0.25-6 mg/mL) in 50 mM citrate buffer (pH 6.0) and incubated at 40 °C for 10 min. The K_m and V_{max} values were then calculated from kinetic data using the Origin 2020 software.

TLC analysis of colloidal chitin hydrolysates produced by the recombinant chitinases

The standard enzyme reaction mixtures were assayed under the standard condition, and

525 then stopped by boiling for 10 min. After centrifugation, the supernatants of mixtures
526 were spotted onto an HSGF254 silica gel plate (Yantai Jiangyou silica gel Development
527 Co., Ltd, China), or were concentrated about 15 times by a vacuum centrifuge and then
528 spotted on the plate. TLC plates were developed with a solvent system containing
529 isopropyl alcohol, water, and 25% ammonia solution [20:5:1 (v:v:v)], prayed by
530 aniline-diphenylamine reagent (4 mL of aniline, 4 g of diphenylamine, 200 mL of
531 acetone, and 30 mL of 85% phosphoric acid)[8], and then baked at 150 ℃ for 5-10 min.
532 The hydrolysates were estimated using the chitooligosaccharides standard, a product of
533 Tokyo Chemical Industry Co., Ltd., Japan.

534 **Additional file**

535 One additional file containing the nucleic acid and amino acid sequences of P1724 was
536 provided.

537 **Ethics approval and consent to participate**

538 Not applicable.

539 **Consent for publication**

540 Not applicable.

541 **Availability of data and materials**

542 The datasets supporting the conclusions of this article are included within the article and
543 its additional files.

544 **Competing interests**

545 The authors declare that they have no competing interests

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Authors' contributions

YD designed and carried out most of the experiments and drafted the manuscript; FY, XL, and HW did and assisted part of the experiments, ZY designed experiment, and revised and proofed the manuscript. All authors read and approved the final manuscript.

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682 Fig. 1. Domain structures of P1724 and truncated portions of P1724 created for this
683 study.
684 Fig. 2. Phylogenetic tree analysis of two GH18 domains of P1724.
685 Tree scale: 0.1 substitutions per nucleotide position.
686 Fig. 3. SDS‑PAGE, Native PAGE, and zymogram analysis of the purified recombinant
687 chitinases.
688 a. SDS-PAGE stained by Coomassie blue. b. Native PAGE stained by Coomassie
689 blue. c. Native PAGE with 0.1% glycol chitin stained by calcofluor white M2R
690 (zymogram). M, protein molecular mass marker, from top to bottom: 200, 116, 97.2,
691 66.4, and 44.3 KDa; Band i, P1724, band ii P1724nGH18 and band iii P1724cGH18.
692 Fig. 4. Effects of temperature and pH on three recombinant chitinases.
693 a. Optimum temperature. b. Temperature stability. c. Optimum pH. d. pH stability.
694 Fig. 5. Effects of NaCl on three recombinant chitinases.
695 Fig. 6. Effects of metal irons and reducing agents on three recombinant chitinases.
696 Fig. 7. TLC analysis of colloidal chitin hydrolysates produced by three recombinant
697 chitinases.
698 a. hydrolysis products without being concentrated. b. hydrolysis products
699 concentrated 15 times. Lane Standard, G1-G6: GlcNAc - $(GlcNAc)_6$.
700
701

Figures

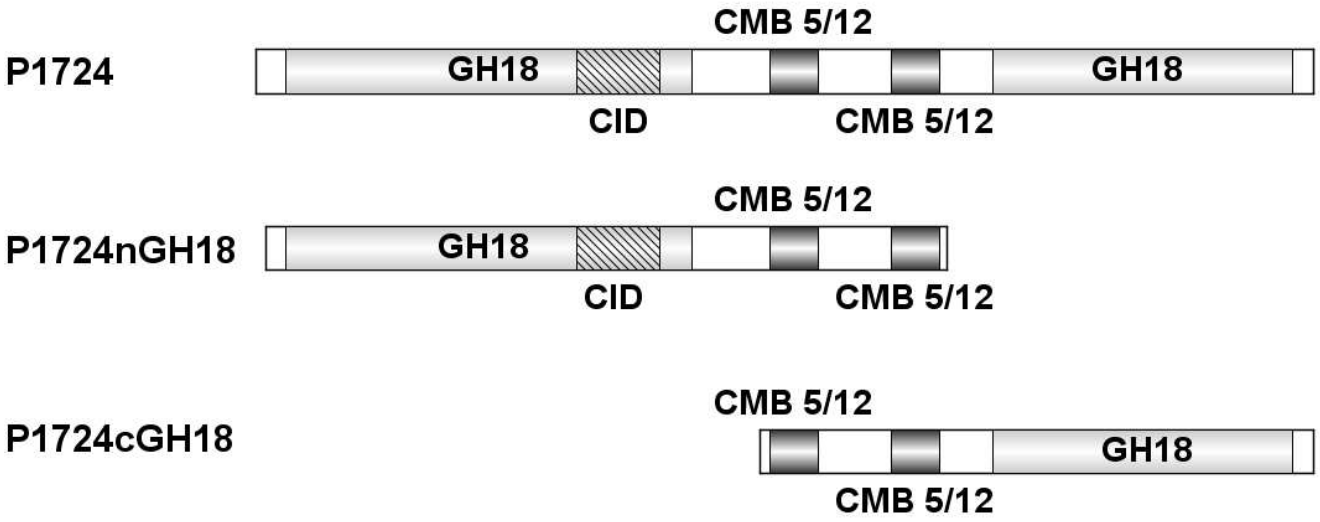

Figure 1

Domain structures of P1724 and truncated portions of P1724 created for this study.

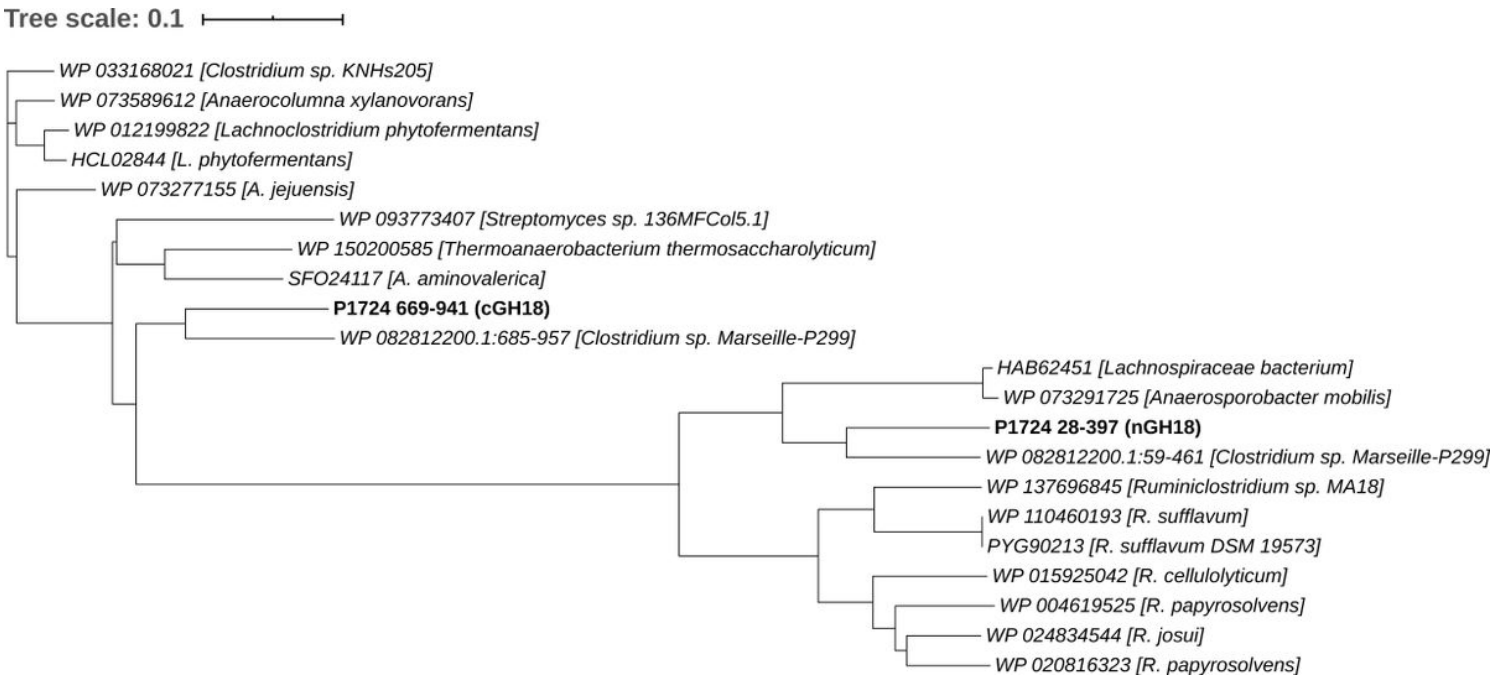

Figure 2

Phylogenetic tree analysis of two GH18 domains of P1724. Tree scale: 0.1 substitutions per nucleotide position.

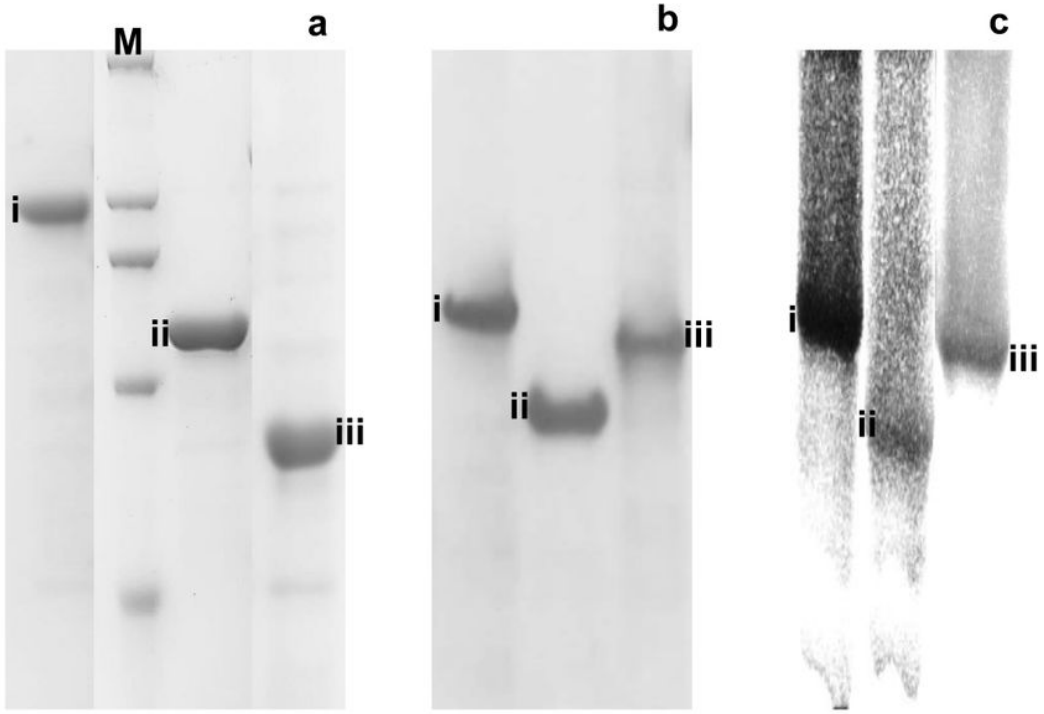

Figure 3

SDS‐PAGE, Native PAGE, and zymogram analysis of the purified recombinant chitinases. a. SDS-PAGE stained by Coomassie blue. b. Native PAGE stained by Coomassie blue. c. Native PAGE with 0.1% glycol chitin stained by calcofluor white M2R (zymogram). M, protein molecular mass marker, from top to bottom: 200, 116, 97.2, 66.4, and 44.3 KDa; Band i, P1724, band ii P1724nGH18 and band iii P1724cGH18.

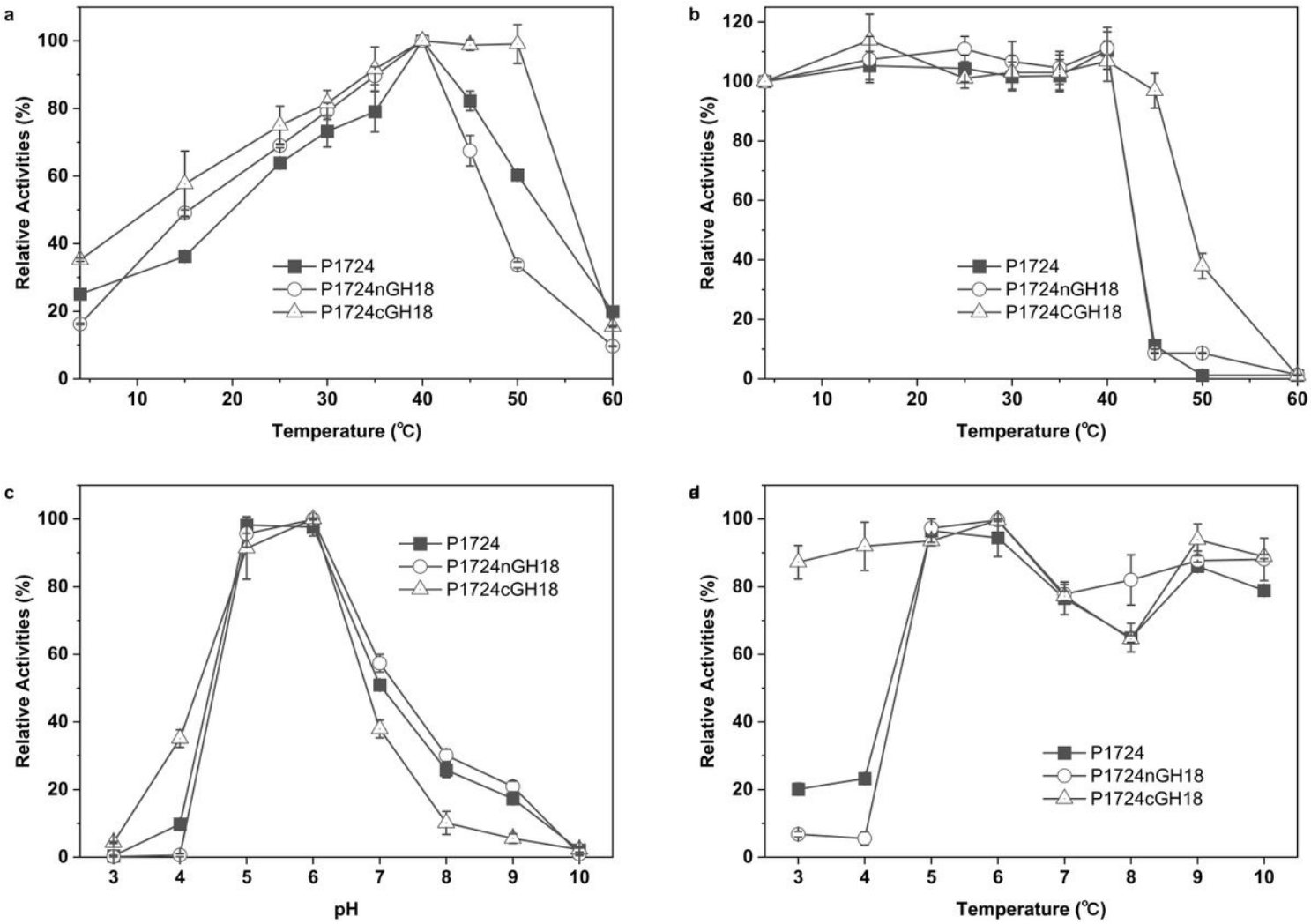

Figure 4

Effects of temperature and pH on three recombinant chitinases. a. Optimum temperature. b. Temperature stability. c. Optimum pH. d. pH stability.

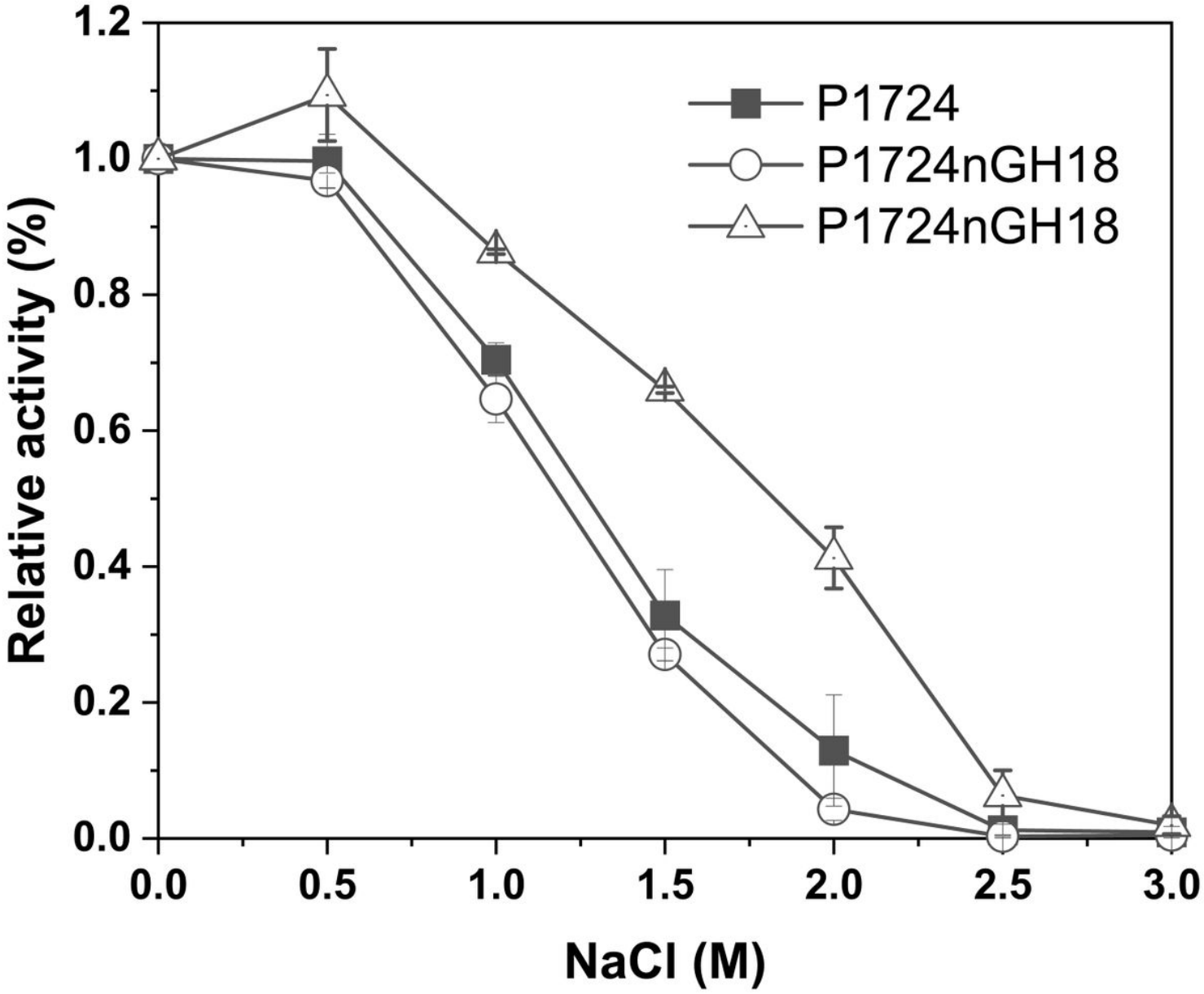

Figure 5

Effects of NaCl on three recombinant chitinases.

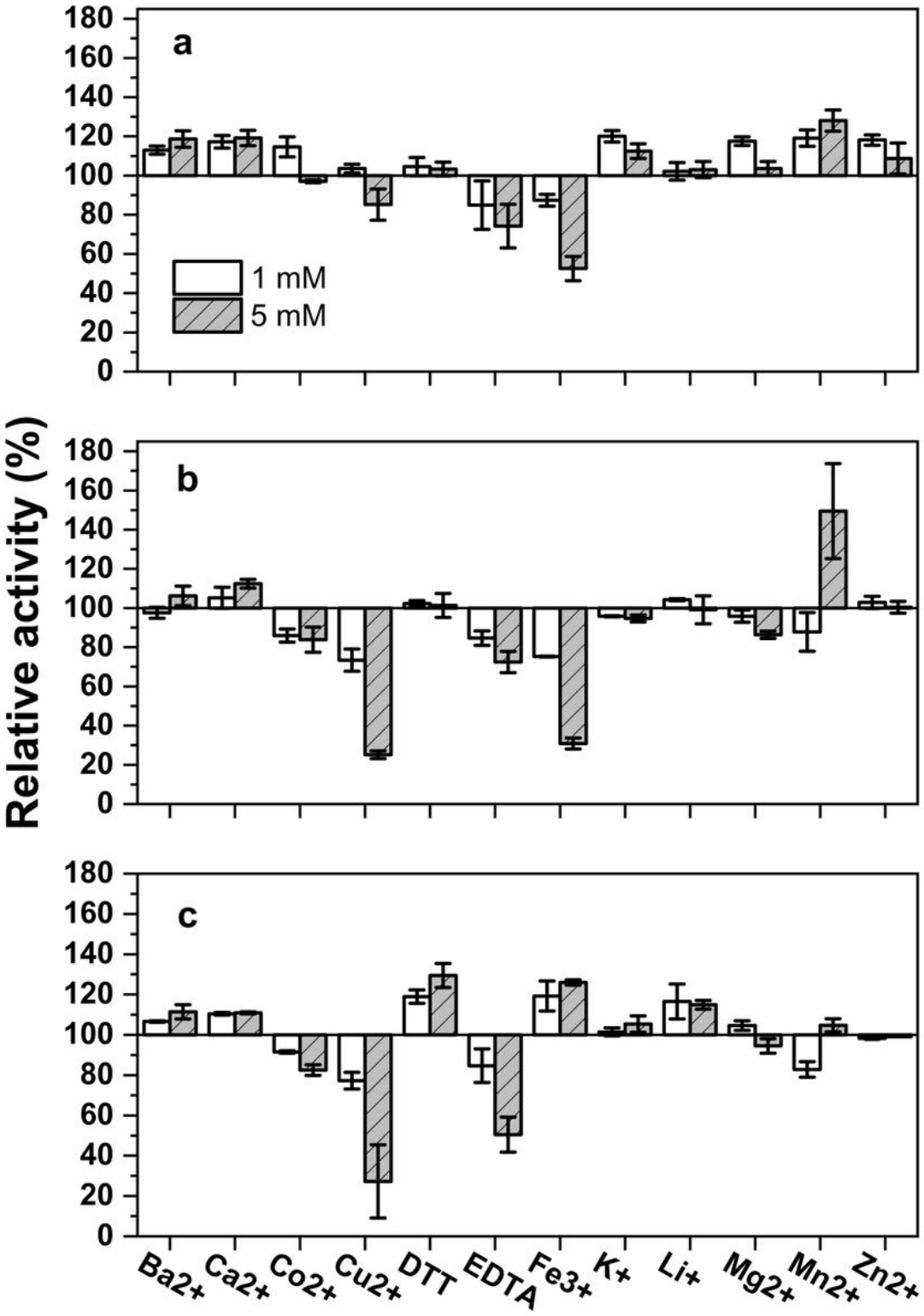

Figure 6

Effects of metal irons and reducing agents on three recombinant chitinases.

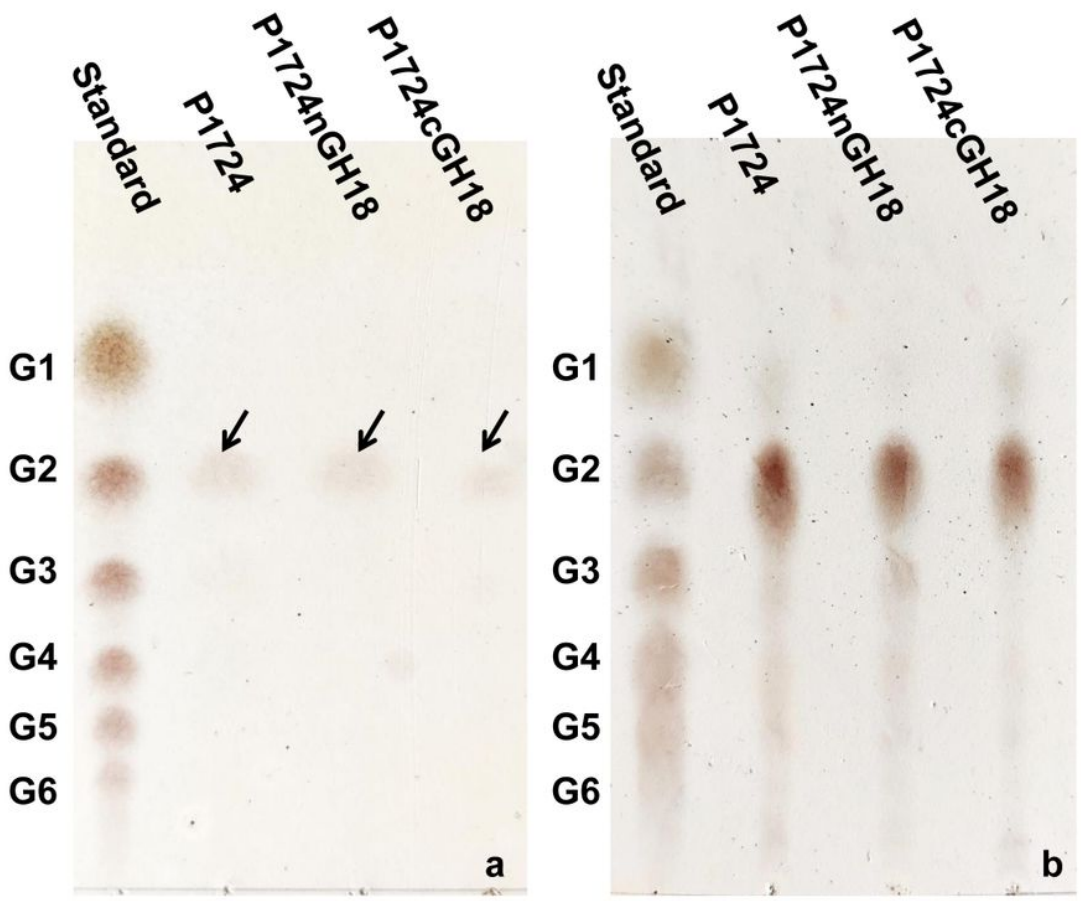

Figure 7

TLC analysis of colloidal chitin hydrolysates produced by three recombinant chitinases. a. hydrolysis products without being concentrated. b. hydrolysis products concentrated 15 times. Lane Standard, G1-G6: GlcNAc - (GlcNAc)6.

Supplementary Files

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