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Purification and Characterization of Two Endo-β-1,4-glucanases from Mollusca, *Ampullaria crossean*

Yan-Hong LI[1#], Rui GUO[3#], Qiu-Yu YIN[1], Ming DING[1], Si-Liang ZHANG[3], Gen-Jun XU[1,2], and Fu-Kun ZHAO[1,2*]

[1] *Key Laboratory of Proteomics, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Graduate School of the Chinese Academy of Sciences, Chinese Academy of Sciences, Shanghai 200031, China;*
[2] *College of Life Science, Zhejiang Sci-Tech University, Hangzhou 310018, China;*
[3] *Bioengineering Institute, East China University of Science and Technology, Shanghai 200233, China*

Abstract Two novel endo-β-1,4-glucanases, EG45 and EG27, were isolated from the gastric juice of mollusca, *Ampullaria crossean*, by anion exchange, hydrophobic interaction, gel filtration and a second round of anion exchange chromatography. The purified proteins EG45 and EG27 appeared as a single band on sodium dodecylsulfate polyacrylamide gel electrophoresis with a molecular mass of 45 kDa and 27 kDa, respectively. The optimum pH for CMC activity was 5.5 for EG45 and 4.4–4.8 for EG27. The optimum temperature range for EG27 was broad, between 50 ºC and 60 ºC; for EG45 it was 50 ºC. The analysis on the stability of these two endo-β-1,4-glucanases showed that EG27 was acceptably stable at pH 3.0–11.0 even when the incubation time was prolonged to 24 h at 30 ºC, whereas EG45 remained relatively stable at pH 5.0–8.0. About 85% of the activity of EG27 could be retained upon incubation at 60 ºC for 24 h. However, less than 10% residual activity of EG45 was detected at 50 ºC. Among different kinds of substrates, both enzymes showed a high preference for carboxymethyl cellulose. EG45, in particular, showed a carboxymethyl cellulose hydrolytic activity of 146.5 IU/mg protein. Both enzymes showed low activities to xylan (from oat spelt) and Sigmacell 101, and they were inactive to p-nitrophenyl-β-*D*-cellobioside, salicin and starch.

Key words cellulase; endo-β-1,4-glucanase; *Ampullaria crossean*; purification; substrate specificity

Today, cellulases and hemicellulases are commonly used in many industrial applications, especially in textile, food, brewing and wine-making as well as in the pulp and paper industries [1,2]. With the shortage of fossil fuels, the emission of greenhouse gases and air pollution by incomplete combustion of fossil fuel, there has been increasing worldwide interest in the production of bioethanol from lignocellulosic biomass [3,4]. To utilize those materials and to avoid waste pollution, one of the most important approaches is to find applicable cellulases and hemicellulases to hydrolyze the lignocellulosic biomass [5,6]. However, the cost of enzymes involved in the production of bioethanol might be an obstacle [3]. Therefore, there is a perpetual interest in finding new, stable and highly efficient cellulases and hemicellulases to meet industrial requirements.

Many microorganisms have been reported as good sources for the production of cellulases and hemicellulases. The soft rot fungus *Trichoderma reesei* has been studied in detail due to its ability to secrete large amounts of enzymes (up to 35 g per liter) [7]. In recent years, owing to the unique characteristics of animal cellulases, a number of laboratories have paid great attention to finding animal sources for cellulases, for example, root-knot nematode *Meloidogyne incognita* [8], crayfish [9], blue mussel [10], abalone [11], beetle [12], and *A. crossean* [13]. We have reported a multifunctional cellulase EGX from the gastric juice of *A. crossean*. The cellulolytic and hemicellulolytic

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# These authors contributed equally to this work
*Corresponding author: Tel, 86-21-54921155; Fax, 86-21-54921011, E-mail, fkzhao@sibs.ac.cn

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enzymes in mollusca, *A. crossean*, have not been investigated systematically. It is worthwhile identifying new enzymes from this species.

Cellulases are responsible for the hydrolysis of the β-1, 4-glucosidic bonds in cellulose. Ordinarily it was accepted that effective biological hydrolysis of cellulose to glucose requires synergistic collaboration of three different kinds of enzymes: endo-β-1,4-glucanase (EC 3.2.1.4, EG) which randomly cleaves internal linkages in cellulose chains; cellobiohydrolase (EC 3.2.1.91, CBH) which specifically cleaves cellobiosyl units from non-reducing ends of cellulose chains; and β-*D*-glucosidase (EC 3.2.1.21) which cleaves glucosyl units from cellooligo-saccharides [14].

This study focused on the purification of two novel cellulases from the gastric juice of *A. crossean*. In addition, different substrates were used to analyze the substrate specificity of the purified enzymes. Other biochemical properties of these two enzymes were also studied.

Materials and Methods

Materials

A. crossean was purchased locally (Xiamen, China). Carboxymethyl cellulose (CMC, medium viscosity), Sigmacell 101, xylan, starch and p-nitrophenyl-β-*D*-cellobioside (pNPC) were purchased from Sigma Chemical (St. Louis, USA), Avicel pH101 was purchased from Fluka (Buchs, Switzerland), DEAE-Sepharose CL-6B, phenyl-Sepharose CL-4B and DEAE-Sepharose fast flow were bought from Pharmacia AB (Uppsala, Sweden). Bio-gel P-100 was from Bio-Rad Laboratory (Hercules, USA). The other chemicals used were of reagent grade and from Shanghai Chemical Industries (Shanghai, China).

Enzyme activity assay

Endo-β-1,4-glucanase activity is determined by the hydrolysis of 200 μl 1% CMC in 100 mM sodium acetate buffer containing 100 mM NaCl (pH 5.2) at 50 ºC for 10 min. Dinitrosalicylic acid (0.5 ml) was added to stop the reaction by boiling in a water bath for 5 min and quick cooling to room temperature. The absorbance at 540 nm was measured (as standard assay). One unit of endo-β-1, 4-glucanase activity is defined as the amount of enzyme that yields 1 μmol glucose in 1 min at 50 ºC.

Determination of protein concentration

Protein concentration was determined by Lowry's method [15], using bovine serum albumin as a standard.

Purification of EG45

The purification of EG45 was carried out at 4 ºC. The *A. crossean* stomachs (18 g) were cut into small pieces, blended in 24 ml (1:1.5, *W/V*) of buffer A (**Table 1**), and centrifuged at 12,000 rpm for 15 min. The supernatant was adjusted to 60% saturation and stood overnight. The precipitate was centrifuged at 12,000 rpm for 15 min and dissolved in buffer A and dialyzed against buffer A, then applied onto a DEAE-Sepharose fast flow column (2.6 cm×16 cm) pre-equilibrated with buffer A and eluted with buffer A. The eluted fractions with CMC activity were collected and changed to buffer C (**Table 1**), then applied onto a phenyl-Sepharose CL-4B column (2.0 cm×20 cm) pre-equilibrated with buffer C. The column was eluted with a linear gradient from 170 ml buffer C to 170 ml buffer B (**Table 1**), and subsequently with a linear gradient from buffer B to buffer A, each 200 ml, then eluted with buffer A. The small peak with CMC activity appeared in the first gradient, which corresponds to EG27. The large peak with cellulase activity appeared in the second gradient, which was concentrated with Ultrafilter PM10 (Millipore Corporation, Bedford, USA) and applied to a Bio-gel P-100 column (2.7 cm×92 cm) pre-equilibrated with buffer A. The enzyme was eluted with buffer A. The large peak with enzyme activity was concentrated and dialyzed with buffer D (**Table 1**). The enzyme was applied to a DEAE-Sepharose fast flow column (1.0 cm×6.0 cm) pre-equili-

Table 1 Buffers used for the purification of enzymes EG45 and EG27

Buffer	Composition
A	10 mM Na_2HPO_4-NaH_2PO_4, pH 6.8, 100 mM NaCl, 1 mM EDTA
B	50 mM NaAC-HAC, pH 5.2, 0.1 M NaCl, 1 mM EDTA
C	50 mM NaAC-HAC, pH 5.2, 0.5 M $(NH_4)_2SO_4$, 0.1 M NaCl
D	50 mM Tris-HCl, pH 8.0
E	20 mM Na_2HPO_4-NaH_2PO_4, pH 7.5, 1 mM EDTA
F	20 mM Na_2HPO_4-NaH_2PO_4, pH 7.0, 0.75 M $(NH_4)_2SO_4$, 1 mM EDTA
G	20 mM Na_2HPO_4-NaH_2PO_4, pH 7.0, 1 mM EDTA
H	50 mM Tris-HCl, pH 8.2

brated with buffer D and eluted by a gradient of NaCl from 0 M to 0.2 M in buffer D. EG45 was concentrated with Ultrafilter PM10.

Purification of EG27

The purification procedure for EG27 was similar to that for EG45: anion exchange, hydrophobic interaction, gel filtration and a second round of anion exchange chromatography, consecutively, except that different buffers were used. Buffer E (**Table 1**) was used for extraction and first chromatography (DEAE-Sepharose CL-6B), and buffer F (**Table 1**) was used in the next step. The enzyme was further purified by Bio-gel P-100 column with buffer G (**Table 1**). Buffer H (**Table 1**) was used for the final purification.

Electrophoresis and gel diffusion assay

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli [16], and native PAGE was carried out according to routine procedures with 10% or 15% gel at 4 ºC. Proteins were stained with Coomassie brilliant blue R-250 or silver nitrate, and cellulase activity was measured by the gel diffusion method. After native PAGE, the gel was transferred onto an agarose plate containing 0.1% CMC. After being incubated at 45 ºC for 1 h, the plate was stained with 0.1% Congo red for 30 min. The stained gel was finally washed with 1 M NaCl to detect enzyme activity [17].

Optimum pH and temperature for activity and stability

Aliquots of appropriate amounts of enzymes were incubated with 200 μl 1% CMC at 50 ºC and different pH levels: 100 mM citric acid-Na_2HPO_4 of pH 3.0–8.0, for EG45; 100 mM citric acid buffer of pH 3.0, 100 mM acetate buffer of pH 4.0–6.0, and 100 mM phosphate buffer of pH 7.0–8.0, for EG27). To establish the optimum temperature for CMC activity, the enzymes were incubated with 1% CMC for 10 min at different temperatures in the range of 25 ºC to 70 ºC. The reaction was stopped by 0.5 ml dinitrosalicylic acid.

The pH stability was studied by incubating the enzyme at different pH levels: 100 mM KCl-HCl, pH 1.0–2.0; 100 mM Tris-HCl, pH 9.0; 100 mM Na_2CO_3-$NaHCO_3$, pH 10.0; 100 mM Na_2HPO_4-NaOH, pH 11.0; 100 mM KCl-NaOH, pH 12.0–13.0; and pH 3.0–8.0 buffer, the same as the above buffer used for EG27, at 30 ºC for 24 h. The temperature stability measurement was performed by incubating the enzyme at different temperatures in 100 mM acetate buffer (pH 4.8 for EG27; pH 5.2 for EG45) for 24 h. The enzyme activity was measured as described above.

Substrate specificity

Substrate specificity of the purified glucanases was determined by measuring the sugars reduced from CMC (1%), Sigmacell 101 (1%), oat spelt xylan (1%), birchwood xylan (0.5%), potato starch (0.5%), salicin (0.5%) or the p-nitrophenol released from pNPC (0.9 mM) under standard conditions.

Results

Purification of enzymes

EG45 and EG27 were purified from the gastric juice of mollusca, *A. crossean*, by ammonium sulfate fractionation, anion exchange, hydrophobic interaction, gel filtration and a second round of anion exchange chromatography, consecutively (**Fig. 1**). CMC hydrolytic activity was assayed at each step. The final purified enzymes all appeared as a single band on SDS-PAGE. EG45 was purified 38.7-fold with a specific activity of 146.5 IU/mg against CMC and recovery was 1.48% (**Table 2**). EG27 was purified 12.2-fold in a rather low yield. Its specific activity

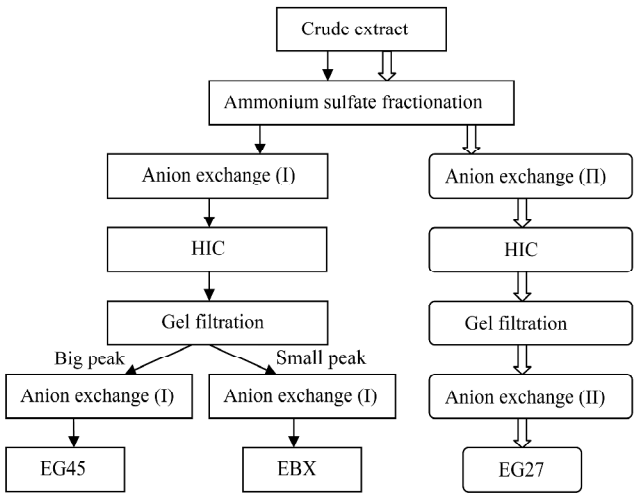

Fig. 1 Purification process of enzymes EG45, EBX and EG27 from the gastric juice of mollusca, *Ampullaria crossean*

Anion exchange (I), DEAE-Sepharose fast flow; anion exchange (II), DEAE-Sepharose CL-6B. EBX is a partially purified enzyme with carboxymethyl cellulose, p-nitrophenyl-β-*D*-cellobioside (pNPC) and xylan hydrolytic activity (data not shown). HIC, hydrophobic interaction chromatography.

Table 2 Purification of enzymes EG45 and EG27 from the gastric juice of mollusca, *Ampullaria crossean*

Purification step	Total protein (mg)	Total activity (U)	Specific activity (IU/mg)	Recovery (%)	Purification fold
EG45					
Extract supernatant	703.4	2666.5	3.79	100	1
Ammonium sulfate	570.3	2299.0	4.03	86.2	1.06
DEAE-Sepharose fast flow	324.7	1689.1	5.20	63.3	1.37
Phenyl-Sepharose CL-4B	41.6	451.2	10.9	16.9	2.88
Bio-gel P-100	4.02	83.79	20.8	3.14	5.49
DEAE-Sepharose fast flow	0.27	39.57	146.5	1.48	38.7
EG27					
Extract supernatant	979.5	2433.1	2.48	100	–
Ammonium sulfate	734.6	1682.2	2.29	69.1	–
DEAE-Sepharose fast flow	132.0	118.8	0.90	4.88	1
Phenyl-Sepharose CL-4B	2.20	3.83	1.74	0.16	1.93
Bio-gel P-100	0.20	1.39	6.97	0.06	7.74
DEAE-Sepharose fast flow	0.06	0.66	11.0	0.03	12.2

"–", the extract supernatant and the ammonium sulfate precipitate contained many kinds of fractions with carboxymethyl cellulose activity, so that it could not be used to estimate the purification fold of EG27 and the specific CMC activity of these two steps was higher than the third step.

against CMC was 11.0 IU/mg (**Table 2**). The molecular mass was 45 kDa for EG45 and 27 kDa for EG27 (**Fig. 2**). Both enzymes exhibited activities on the native PAGE gels by staining with 0.1% Congo red (data not shown).

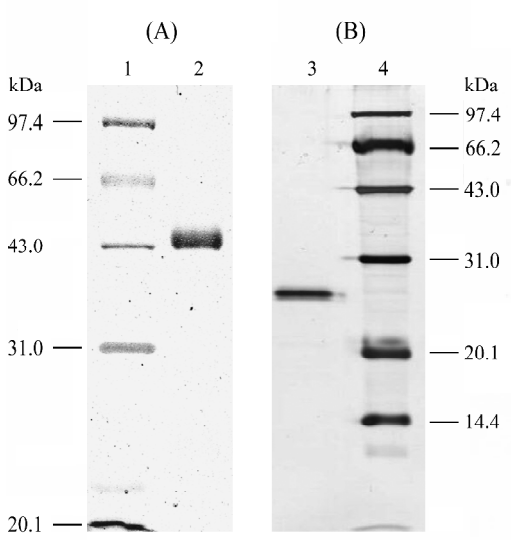

Fig. 2 Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of purified enzymes EG45 and EG27 from the gastric juice of mollusca, *Ampullaria crossean*

1 and 4, molecular mass standards consisting of rabbit phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), rabbit actin (43 kDa), bovine carbonic anhydrase (31 kDa), trypsin inhibitor (20.1 kDa) and lysozyme (14.4 kDa); 2, purified EG45; 3, purified EG27. Gel A (10% SDS-PAGE) was stained with Coomassie brilliant blue R-250. Gel B (15% SDS-PAGE) was stained with silver nitrate.

Determination of optimum pH and temperature

Both of the enzymes showed the highest hydrolytic activity against CMC under acidic conditions. EG45 exhibited maximum activity at pH 5.5, while the activity of EG27 reached its maximum at pH 4.4–4.8. There was a comparatively great decrease in the activity of two enzymes, respectively. At pH 4.0, EG27 retained over 70% activity while EG45 was almost inactive. Under alkaline conditions, both EG45 and EG27 had approximately 20% activity at pH 7.0 and were inactive at pH 8.0 (**Fig. 3**).

EG45 had an unusually sharp curve in optimum temperature. At 50 ºC, the enzyme catalyzed the hydrolysis of CMC efficiently. Above 50 ºC and below 45 ºC, the activity of the enzyme declined rapidly. A unique characteristic of EG27 was its broad optimum activity temperature range, between 50 ºC and 60 ºC, and about 90% of its maximum activity was obtained at 45 ºC (**Fig. 4**).

Effect of pH and temperature on enzyme stability

The pH stability was determined by measuring the residual activity after incubation of the enzyme at 30 ºC for 24 h in buffers with differing pH. EG27 was stable in a broad range of pH 3.0–11.0, and more than 80% maximum activity of EG45 was observed at pH 5.0–8.0. In addition, EG27 showed an unusual, almost acidophilic and alkaliphilic feature in that it retained more than 70% of its maximum activity at pH 12.0 and more than 40% at pH

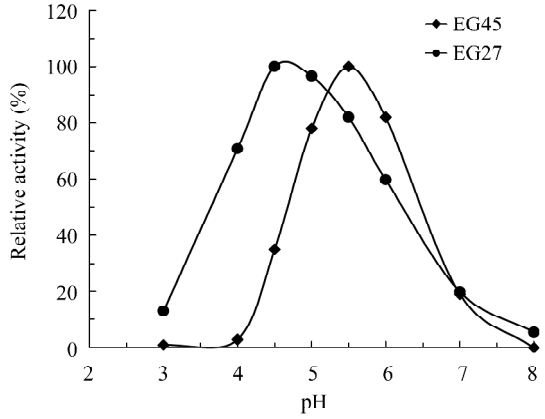

Fig. 3 Effect of pH on the activity of enzymes EG45 and EG27

Relative enzyme activities (% of maximum) of EG45 and EG27 at different pH levels are shown. Enzyme activity was assayed by incubating the enzyme with the carboxymethyl cellulose substrate at different pH levels for 10 min at 50 ºC. The reducing sugars produced were determined by the standard assay.

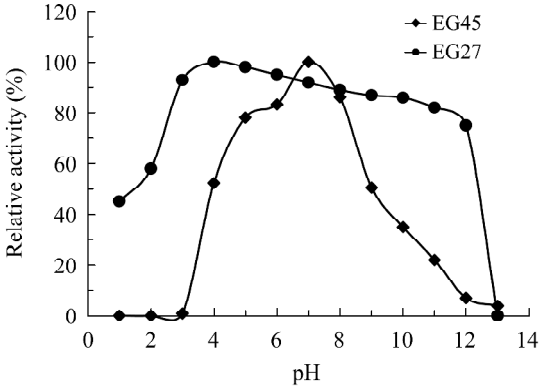

Fig. 5 Effect of pH on the stability of enzymes EG45 and EG27

The enzymes EG45 and EG27 were incubated in buffers of differing pH for 24 h at 30 ºC prior to measuring the residual carboxymethyl cellulose activity under the standard assay conditions.

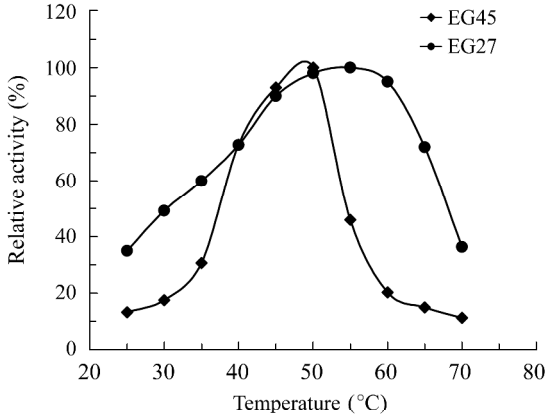

Fig. 4 Effect of temperature on the activity of enzymes EG45 and EG27

Relative enzyme activities (% of maximum) of EG45 and EG27 at temperatures ranging from 25 ºC to 70 ºC with carboxymethyl cellulose as the substrate. The reducing end groups exposed were determined using the dinitrosalicylic acid method.

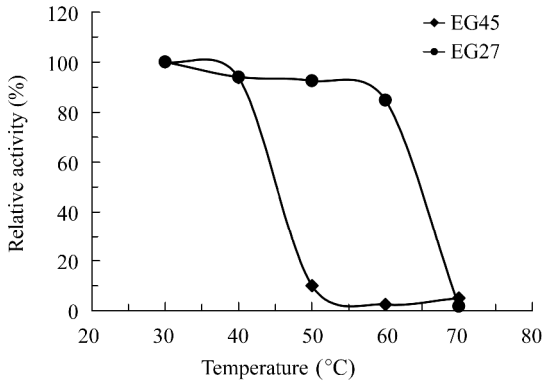

Fig. 6 Effect of temperature on the stability of enzymes EG45 and EG27

The enzymes EG45 and EG27 were incubated in 100 mM acetate buffer, pH 4.8 (EG27) or pH 5.2 (EG45) at temperatures ranging from 25 ºC to 70 ºC for 24 h prior to measuring the carboxymethyl cellulose activity as the standard assay.

1.0 after incubation at 30 ºC for 24 h. However, EG45 expressed only about 20% activity at pH 12.0 and was inactive below pH 3.0 (**Fig. 5**).

In a separate temperature stability study (the remaining activity was measured with CMC as the substrate), we found that EG27 exhibited high thermostability. About 85% of the enzyme activity was retained when the incubation time was even prolonged to 24 h at 60 ºC. As far as EG45 was concerned, less than 10% activity was retained at 50 ºC. Both enzymes were completely inactive after incubation at 70 ºC for 24 h (**Fig. 6**).

Substrate specificity

Various substrates, such as CMC, Sigmacell 101, xylan, starch, salicin and pNPC, were used to analyze the substrate specificity of EG45 and EG27, and results are shown in **Table 3**. EG45 had much higher specific activity against CMC than EG27. The CMC hydrolytic activity of EG45 was 146.5 IU/mg and EG27 was 11.0 IU/mg. Apart from CMC, EG45 and EG27 were found to hydrolyze oat spelt xylan (8.1 IU/mg and 0.5 IU/mg, respectively) and showed low activity towards microcrystalline cellulose Sigmacell 101 (0.8 IU/mg and 0.2 IU/mg, respectively). EG45 also had lower hydrolytic activity towards xylan from birch wood; EG27 showed none. No activity was found with

Table 3 Activity of enzymes EG45 and EG27 on various substrates

Substrate	Enzyme activity (IU/mg)	
	EG45	EG27
CMC	146.5±1.1	11.0±0.6
Oat spelt xylan	8.1±0.3	0.5±0.1
Sigmacell 101	0.8±0.1	0.2±0.1
Birch wood xylan	0.8±0.1	0
pNPC	0	0
Potato starch	0	0
Salicin	0	0

CMC, carboxymethyl cellulose; pNPC, p-nitrophenyl-β-*D*-cellobioside.

pNPC, starch or salicin for either of these two enzymes. These results suggested that both EG45 and EG27 were typical endo-β-1,4-glucanases.

Discussion

A. crossean is a kind of herbivorous mollusca. The activities of exo-β-1,4-glucanase, endo-β-1,4-glucanase and β-glucosidase have been detected in the gastric juice of *A. crossean* [18]. In a previous paper, we reported a cellulase named EGX which belongs to the glycosyl hydrolase family 10 (GHF10) and is a multifunctional cellulase with exo-β-1,4-glucanase, endo-β-1,4-glucanase and endo-β-1,4-xylanase [18]. Furthermore, the endogenous origins of the *EGX* gene [13] and a kind of endo-β-1,4-glucanase gene (*EG65*) (data not shown) were confirmed by PCR amplification from the ovary genomic DNA of the mollusca. Here two novel endo-β-1,4-glucanases, EG45 and EG27, were purified from the gastric juice of the mollusca. Although the origins of EG45 and EG27 remain to be confirmed, genes encoding cellulase and xylanase at least existed in the mollusc itself. These results indicated that *A. crossean* might have its own intact cellulase system.

Although optimal pH values for cellulase activity vary from acidic to alkaline [19–22], all animal cellulases reported until now have optimal activity under weak acidic conditions [23–25]. The optimal pH for purified EG45 and EG27 from the gastric juice of the mollusca, *A. crossean*, against CMC was also 5.5 and 4.4–4.8, respectively. This is in accordance with the acidic environmental conditions of the stomach. In some biotechnological applications, some processes need acidic enzymes to hydrolyze the cellulose materials. Currently, a combination of pectinases, cellulases and hemicellulases, collectively called macerating enzymes, are used in the extraction and clarification of fruit and vegetable juices [26,27]. α-amylase and amyloglucosidase were active at acidic pH and used to process starch-containing fruits, especially apples harvested early in order to prevent haze formation [27, 28].

In addition to acidophilic property, EG45 and EG27 had their own unique characteristics. EG45 had high activity against CMC-Na. The specific activity reaches 146.5 IU/mg protein which is much higher than some cellulases from other species [11,25,29] and is similar to that reported for purified endoglucanase from the yellow-spotted longicorn beetle, *Psacothea hilaris* [12]. Stability is an important criterion of enzymes, especially for cellulases and hemicellulases with their enormous potential application in biotechnology and industry [1]. The thermostable analysis of EG27 showed that the enzyme exhibited high thermostability. About 85% of the enzyme activity was retained when the incubation time was prolonged to 24 h at 60 ºC. Furthermore, EG27 is stable at a broad pH range (pH 3.0–11.0), and more than 70% of its activity remained after incubation at pH 12.0 for 24 h, at 30 ºC. It is comparable to the endoglucanase from *Bacillus pumilus* that 75% of the activity remained after incubation at pH 12.0 for 20 h, at 30 ºC [30]. Hence, these two novel enzymes would attract active research and commercial interest due to their potential extensive applied value.

Cellulases have been extensively studied on cellulolytic microorganisms [31] and studies of cellulolytic enzymes at the molecular level have revealed some of the features that contribute to their activity. However, the structure and catalytic mechanisms of animal cellulases are poorly understood. Until now, no crystal structure of any animal cellulase has been solved because of glycosylation or other post-translational modifications. Without glycosylation and other post-translational modifications, the low molecular mass of EG27 would be an ideal system easily expressed in *Escherichia coli* and obtained in large quantities for structure and function studies at the molecular level.

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Edited by
Qin-Sheng YUAN