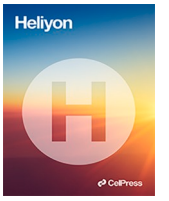
Heliyon 10 (2024) e29643

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Pretreatment of chitin depolymerization by expansin-like protein to improve hydrolysis efficiency

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ARTICLE INFO

Keywords:
Chitin depolymerisation
Chitin accessibility
Expansin-like protein

ABSTRACT

Chitin is a polysaccharide similar to cellulose that contains abundant hydrogen bonds. Expansin-like proteins disrupt hydrogen bond networks, causing cellulose to swell and accelerating its degradation. We examined the effects of pretreatment with two expansin-like proteins, CxEXL22 (*Arthrobotrys* sp. CX1) and HcEXL *(Hahella chejuensis*), on chitin depolymerisation and enzymatic degradation. The efficiency of chitin degradation increased more than two-fold after pretreatment with expansin-like proteins. Following pretreatment with expansin-like proteins, chitin had a lower crystallinity index, greater d-spacing and crystallite size, and weaker hydrogen bonds, and the loosened porous microfibrils were more exposed than in untreated chitin. The rupture characterisation of crystalline chitin indicated that expansin-like proteins loosened the hydrogen bonds of the chitin polysaccharide chains, causing significant depolymerisation to expose more porous structures and enhance chitin accessibility.

1. Introduction

Chitin, a polysaccharide polymer consisting of *N*-acetyl-D-glucosamine linked by β-1, 4 bonds, is the second most abundant biomass resource after cellulose. Chitin degradation products have a variety of functional properties (antioxidant, antibacterial, and anti-inflammatory) and are widely used in agriculture, medicine, food, and cosmetics [1–3]. However, chitin degradation is limited by its high crystallinity and polymerisation. Depending on the chitin orientation, crystalline chitin is found in three distinct forms: α-, β-, and γ-forms, with the α-form being the most prevalent [1]. α-Chitin exhibits high crystallinity due to the arrangement of chitin sheets in an anti-parallel manner, facilitating extensive hydrogen bonding, including two intra-molecular and two inter-molecular bonds [1]. Chitin polymerisation is similar to cellulose polymerisation, and the adjacent polymer chains contain an abundance of hydrogen bonds that form various ordered crystalline arrangements, resulting in a chitin crystalline region with high crystallinity and insolubility [4,5]. The accessibility of chitin is the most important factor affecting the rate and extent of chitin degradation, reflecting the available area for aqueous solution and chitinase to access and hydrolyse the chitin chains. Accessibility is strongly influenced by polymerisation and crystallinity [6,7]. Thus, depolymerisation and decrystallinisation can improve the solubility and accessibility of chitin, and enhance its degradation.

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https://doi.org/10.1016/j.heliyon.2024.e29643
Received 17 February 2024; Received in revised form 10 April 2024; Accepted 11 April 2024
Available online 12 April 2024

Strong acids or ionic liquids are the most commonly used pretreatments for chitin dissolution [8–11]. However, these solvents are generally toxic or corrosive, causing environmental hazards, recovery problems, and high cost of pretreatment, which limits their application. Pretreatment by steam explosion requires a high temperature of 160–260 °C and high-pressure equipment purchase [12]. Enzymatic degradation is an environmentally friendly process that requires simple reaction conditions. Expansins and expansin-like proteins have been identified as plant cell wall-loosening proteins [13,14]. These proteins have been reported to hydrolyse hydrogen bonds between cellulose polysaccharide chains without detectable hydrolytic activity in cooperation with cellulase, thereby improving cellulose hydrolysis [14,15]. The expansin-like proteins CxEXL22 from *Arthrobotrys* sp. CX1 and HcEXL from *Hahella chejuensis* loosen the hydrogen bonds between cellulose polysaccharides, which promotes the accessibility of cellulose and thus enhances cellulose degradation [16,17]. However, there have been few studies on the use of expansin-like proteins to pretreat chitin. In this study, we showed that the use of expansin-like proteins CxEXL22 and HcEXL to pretreat chitin to loosen the hydrogen bonds between polysaccharide chains, led to depolymerisation of the crystalline structure of chitin and an increase in chitinase efficiency. These results suggest that the expansin-like proteins CxEXL22 and HcEXL have potential applications in chitin conversion.

2. Materials and methods

2.1. General information

Chitin was extracted from shrimp shells (Cat. #C7170) and chitinase from *Streptomyces griseus* (Cat. #C6137) were purchased from Sigma–Aldrich. (St Louis, MO, USA). All the chemical reagents used in this study were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All reagents used were of analytical grade, unless otherwise specified.

2.2. Expansin-like protein production

CxEXL22 was expressed and purified as previously reported method [17]. The *HcEXL* gene (GenBank accession number ABC30409.1) was synthesised by Comate Bioscience Co., Ltd. (Changchun, China). HcEXL was produced using as previously described [18].

2.3. The capacity of expansin-like proteins to synergize chitinase activity

Chitin was pretreated with 50 mM sodium acetate buffer (pH 6.0), 5 mg of substrate, and 10 μg of CxEXL22 or HcEXL for 14 h at 50 °C with agitation at 160 rpm. The pH and temperature of the expansin-like proteins were selected based on previous studies [17,18]. The pretreated chitin was washed thrice with distilled water. Chitinase at the final concentration of 0.1 U (one unit liberates 1.0 mg of *N*-acetyl-D-glucosamine from chitin per hour at pH 6.0 at 25 °C in a 2 h assay; one new 1 h unit = approx. 50 old 48-h units) was added to the pretreated chitin at pH 6 for 24 h at 40 °C and agitation at 160 rpm. The simultaneous addition of the expansin-like protein and chitinase in the reaction at pH 6 for 24 h at 40 °C and agitation at 160 rpm was defined as simultaneous treatment (S), and incubation with the expansin-like protein alone for 24 h (pretreatment system) followed by the addition of chitinase was defined as pretreatment (P). Bovine serum albumin (BSA) buffer was used instead of expansin-like protein in the blank sample. The concentration of the released reducing sugars was determined by the DNS method using *N*-acetylglucosamine (NAG) as a calibration standard. For the control, chitinase activity was measured at a final concentration of 0.1U chitinase at pH 6 for 24 h at 40 °C and agitation at 160 rpm using untreated chitin. All assays were performed in triplicates. The degree of chitin activity enhancement is represented by the degree of synergy (DS), which is defined as Eq. (1):

$$DS = \frac{R_{\text{expansin-like protein+chitinase}}}{R_{\text{expansin-like protein}} + R_{\text{chitinase}}} \tag{1}$$

where $R_{\text{expansin-like protein + chitinase}}$ represents the amount of reducing sugar generated by treatments S and P, and $R_{\text{expansin-like protein}} + R_{\text{chitinase}}$ represents the total amount of reducing sugar generated by expansin-like protein and chitinase, respectively [19].

2.4. Analysis with SEM, FTIR and XRD

For microscopic observations, the different reaction mixture samples were washed three times in an ultrasonic bath and then dried at 60 °C for 30 min. The samples were visualised using a scanning electron microscope (SEM; JSM-6460LV, JEOL, Tokyo, Japan). Changes in chemical bonds and functional groups were detected by Fourier-transform infrared spectroscopy (FTIR; Spectrum Two, PerkinElmer, China) in the wavenumber range of 400–4000 cm^{-1}. The degree of acetylation of chitins (DA) and the degree of deacetylation of chitins (DD) were calculated using Eq. (2):

$$DA\ (\%) = \left(A_{1655}/A_{3450}\right) \times \frac{100}{1.33}\ DD\ (\%) = 100 - DA \tag{2}$$

where A_{1655} is the intensity of the peak at wave number 1655 cm^{-1} and A_{3450} is the intensity of the peak at wave number 3450 cm^{-1} [1,20].

The DD of the samples was also determined using a modified acid-base titration method [21]. After desiccation for 24 h at 65 °C,

0.2 g of samples were dissolved in 30 ml of 0.1 M HCl standard solution and stirred for 3 h at 25 °C. Subsequently, the mixture was titrated with 0.1 M NaOH standard solution, utilizing two drops of 0.1 % methyl orange-aniline blue (1:2, V/V) solution as an indicator.

$$DD(\%) = \frac{(30\text{-}V) \times 0.0016 \times 100\%}{m \times 0.0994}$$

where V represents the volume of NaOH at the endpoint of the titration, and m represents the mass of the sample. Experiments were conducted in triplicate and repeated three times.

Changes in chitin crystallinity were determined using X-ray diffraction (XRD; XRD-7000S; Shimadzu, Kyoto, Japan). Data were obtained at 40 kV, 30 mA, 2 θ with a scan angle from 6° to 30° and at a scan rate of 5°/min with a 0.02° step. The chitin crystalline index (CrI) was calculated using Eq. (3):

$$CrI_{020} = \frac{I_{020} - I_{am}}{I_{020}} \times 100 \quad CrI_{110} = \frac{I_{110} - I_{am}}{I_{110}} \times 100. \tag{3}$$

where I_{020} and I_{110} represent the maximum intensities at approximately 10° and 20°, respectively, I_{am} is the intensity of the amorphous diffraction peak at approximately 16°. The d-spacing between the atomic layers and the crystalline size were calculated using Bragg's and Scherrer's equations, respectively [22].

3. Results and discussion

3.1. FT-IR spectra

FTIR has recently been used to characterize subtle changes in the structure and bonding of chitin and its degradation products [4, 22]. Fig. 1 shows the FTIR spectra of untreated chitin and chitin pretreated with the expansin-like proteins CxEXL22 and HcEXL. All samples exhibited the characteristic peaks for α-chitin and no new peak formed during pretreatment with expansin-like proteins. Three peaks of hydrogen bond stretching were present at 3,200–3,500 cm^{-1} representing one intra-chain (3,268 cm^{-1}) and two chitin inter-chain (3,450 and 3,486 cm^{-1}) bonds [8,23]. After pretreatment with expansin-like proteins, the shapes of the peaks of pretreated chitin at approximately 3200–3500 cm^{-1} became broader and smoother, indicating that the hydrogen bonds between chitin polysaccharides were weakened. This indicates that CxEXL22 and HcEXL act on the chitin polysaccharide chains, causing the cleavage of intra- and inter-molecular hydrogen bonds. The DD of non-pretreated chitin was calculated from FTIR data to be 9.66 %, and the DDs of chitin pretreated with the expansin-like CxEXL22 and HcEXL were 13.49 % and 22.54 %, respectively. The DD of samples was determined with a modified acid-base titration method. The DD of non-pretreated chitin was 7.72 ± 0.21 %, and the DDs of chitin pretreated with the expansin-like CxEXL22 and HcEXL were 12.28 ± 0.14 % and 25.33 ± 0.12 %, respectively, which were not significantly different from the DDs calculated by the FTIR method. The DDs of chitin pretreated with the expansin-like proteins CxEXL22 and HcEXL were higher than those of the non-pretreated chitin, which demonstrated that chitin could be partially deacetylated by the expansin-like proteins.

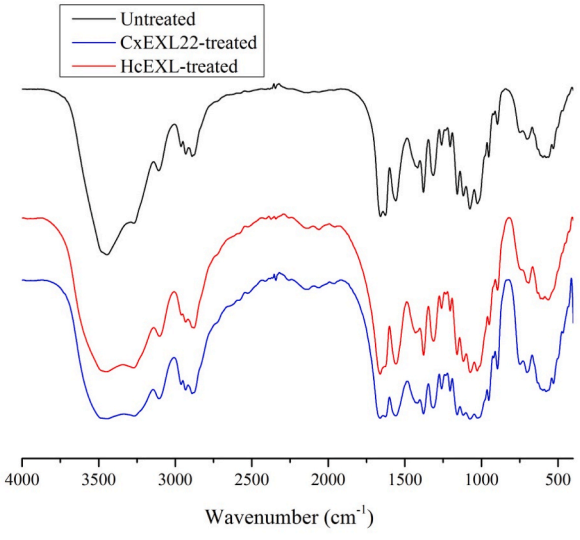

Fig. 1. Comparison of Fourier-transform infrared spectra of chitin after different pretreatments. This shows the hydrogen bond stretching at 3200–3500 cm^{-1}.

3.2. Crystalline characteristics

To evaluate the effect of pretreatment with expansin-like proteins on chitin polymerisation, chitin crystallinity was characterised using XRD. The XRD spectra of the different chitin samples were analyzed as shown in Fig. 2. Five crystal reflections of chitin were observed at approximately 9.3°, 12.6°, 19.2°, 23.3°, and 26.3° for all samples, displaying typical α-chitin patterns [24]. However, compared with untreated chitin, the intensities of the five crystal peaks of pretreated chitin were significantly reduced, resulting in a decrease in CrI. Based on these results, we concluded that the ordered crystalline structure of chitin was disrupted, indicating that the pretreatment of chitin with the expansin-like proteins CxEXL22 and HcEXL loosened the hydrogen bonds between the polysaccharide chains causing chitin decrystallinisation.

Table 1 lists the d-spacing and crystallite size of the (020) and (110) lattice planes of the different chitin samples. The d-spacing changes are based on the inter-molecular forces of molecular chains in chitin microfibrils [25]. The d-spacing showed a slight increase in both pretreated chitin samples compared to that in the untreated chitin, indicating that the inter-molecular forces were weakened during pretreatment with CxEXL22 or HcEXL. Moreover, the crystallite size of chitin significantly increased after pretreatment with CxEXL22 or HcEXL. This indicates that the inter- or intra-molecular hydrogen bonds in the pretreated chitin chains were disrupted and chitin loosened, resulting in an increase in crystallite size and leading to chitin depolymerisation and decrystallisation.

CxEXL22 and HcEXL have previously been reported to disrupt hydrogen bonding between matrix polysaccharides and cellulose microfibrils, causing them to swell [17,18]. After expansin-like protein pretreatment, the crystallinity index of chitin decreased and the d-spacing increased slightly owing to swelling. Concurrently, the increase in the d-spacing led to an increase in crystallite size. This indicates that the hydrogen bonds between the hydroxy groups of the chitin chains weakened, causing the pretreated chitin to swell and disrupt the orderly lattice arrangement. Therefore, there was an increase in the number of hydroxy groups without forming hydrogen bonds, which was supported by the gradual decrease in the FTIR spectra peaks at 3200–3400 cm^{-1}.

3.3. Microstructures

To illustrate the effect of expansin-like proteins on chitin, the microstructural changes in different chitin samples were observed using SEM (Fig. 3). As shown in Fig. 3 A and B, the surface of the untreated chitin displayed tightly arranged overlapping microfibrils, as reported previously [26,27]. This structure is not suitable for aqueous solutions or chitinase to access and hydrolyse the chitin chains. However, after pretreatment with CxEXL22 (Fig. 3 C, D) and HcEXL (Fig. 3 E, F), the chitin microstructure exhibited loosened microfibrils and porous structures, which exposed more microfibrils. These results indicate that the dense structure of chitin was broken and the looseness of chitin chains was significantly increased after pretreatment with expansin-like proteins. Expansin-like proteins hydrolyse the hydrogen bonds between chitin chains, causing chitin depolymerisation. This exposed many more microfibrils and increased the available surface area, thereby enhancing chitin accessibility.

3.4. Synergism between chitinase and expansin-like proteins

Expansin-like proteins hydrolyse the intra- and inter-molecular hydrogen bonds of cellulose molecules to enhance the degradation of cellulose, but their hydrolytic activity cannot be detected alone [28]. To confirm the effect of expansin-like proteins on chitin depolymerisation, the synergism between CxEXL22 or HcEXL and chitinase in chitin degradation was evaluated (Fig. 4). When chitin was used as a substrate for expansin-like proteins and chitinase, it acted synergistically with DS $>$ 1 in simultaneous treatment and pretreatment (Fig. 4A). Moreover, the synergistic effect of pretreatment was stronger than that observed for the simultaneous reaction of chitinase and expansin-like proteins. This finding is consistent with a previous study on the synergism of expansin-like proteins [29].

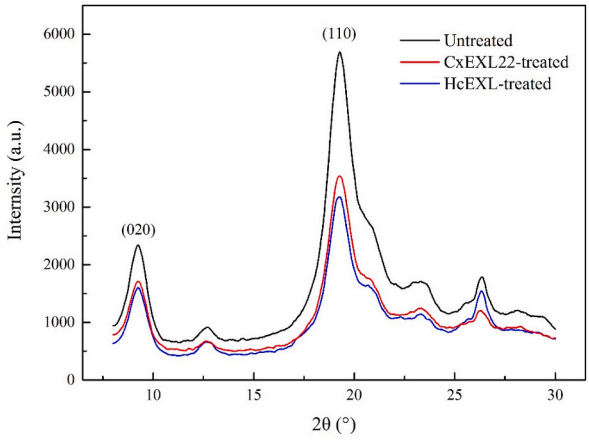

Fig. 2. X-Ray diffraction spectra of chitin after different pretreatments. The intensities of the five crystal peaks of chitin decreased after the pretreatments.

Table 1
Crystallinity characteristics of untreated and expansin-like protein treated chitin samples.

	Crystallinity index (CrI)		d-Spacing (Å)		Crystallinity size (Å)	
	(020)	(110)	(020)	(110)	(020)	(110)
Untreated control	65.7 %	85.9 %	9.53	4.45	94	60
CxEXL22-treated chitin	62.8 %	84.1 %	9.60	4.55	124	74
HcEXL-treated chitin	64.5 %	83.2 %	9.54	4.57	104	75

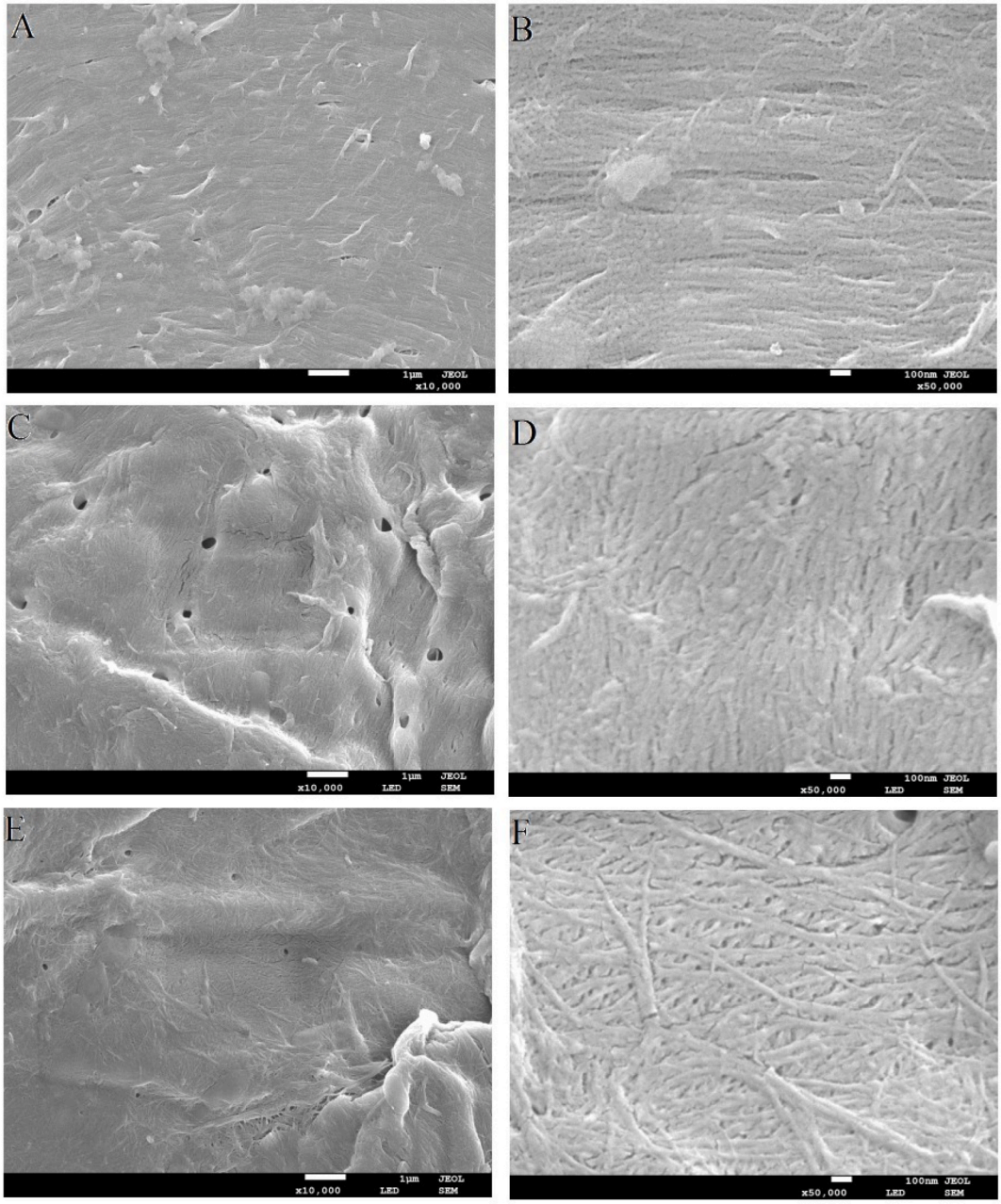

Fig. 3. Scanning electron microscopy images of chitin following different pretreatments (A, B) untreated chitin at (A) 10,000 × and (B) 50,000 × ; (C,D) CxEXL22-treated chitin at (C) 10,000 × and (D) 50,000 × ; (E,F) HcEXL-treated chitin at (E) 10,000 × and (F) 50,000 × . The pretreatments induced ultrastructural changes in chitin.

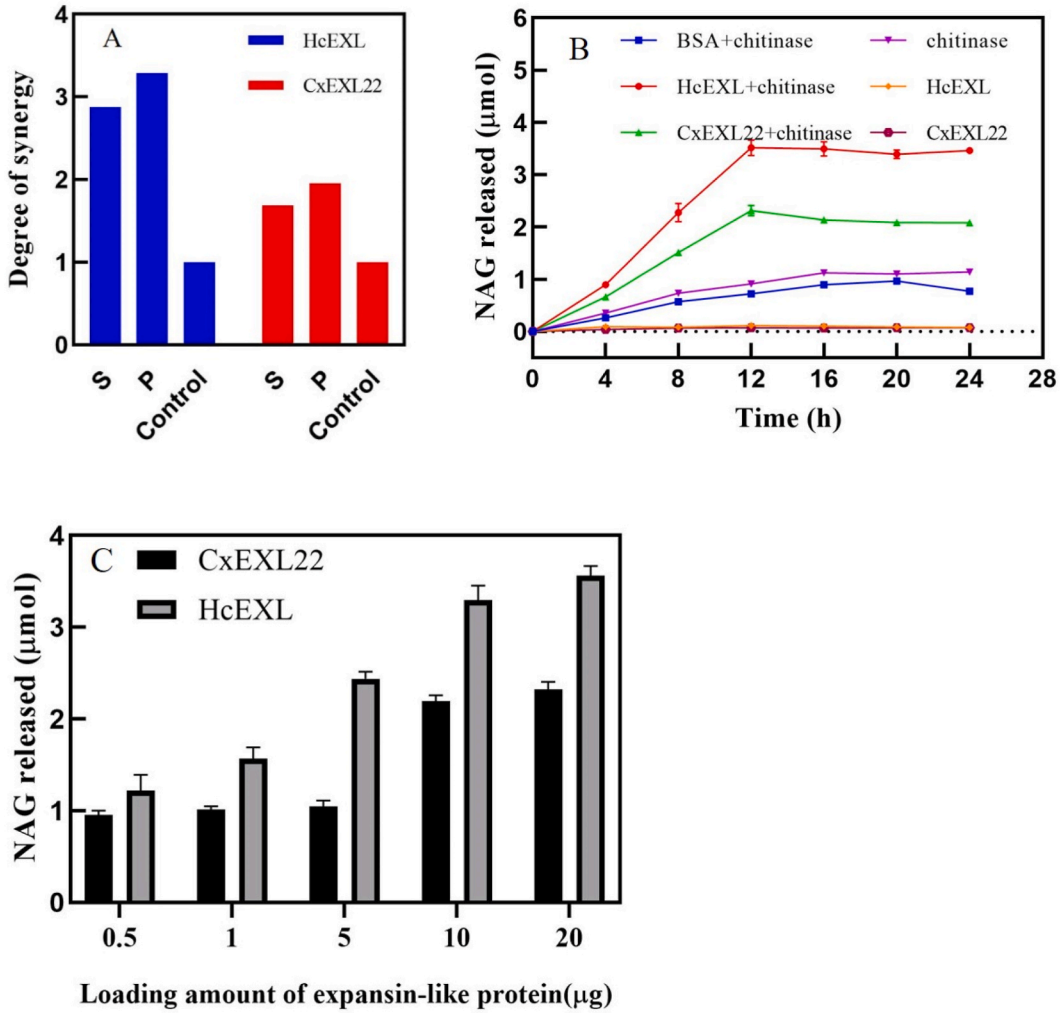

Fig. 4. Synergistic effects of expansin -like proteins and chitinase on chitin hydrolysis. (A) The degree of synergy in simultaneous treatment (S), pretreatment (P) and chitinase alone as the control. (B) The synergistic effects on the hydrolysis of chitin after different pretreatment times. Incubation chitin with 10 μg of CxEXL22 or HcEXL alone for 14 h at 50 °C with agitation at 160 rpm (pretreatment system) followed by the addition of 0.1 U of chitinase at pH 6 for 24 h at 40 °C. (**C**) Effect of expansin-like protein loading on synergism with chitinase. These mean that expansin -like proteins efficiently synergize with chitinase to promote chitin degradation.

As previously reported, incubation of chitin with CxEXL22 or HcEXL alone did not result in significant production of reducing sugars [17,18]. Compared to non-pretreated chitin, chitinase activity increased by 2- and 3.4-fold, respectively, after chitin was pretreated with CxEXL22 or HcEXL for 14 h (Fig. 4B). No differences in NAG yield were observed between the samples treated with BSA (negative control) and those treated with chitinase alone. These results indicate that CxEXL22 and HcEXL hydrolyse chitin synergistically with chitinase to increase chitin degradation efficiency. The amount of NAG released by chitinase hydrolysis significantly increased as the loading of expansin-like proteins increased from 0.5 to 10 μg (Fig. 4C). However, when the amount of expansin-like protein was 20 μg, the increase in the NAG yield released became less significant. This indicated that the synergistic effect became saturated as the quantity of expansin-like proteins increased.

4. Conclusions

The crystallinity and polymerisation of chitin are critical factors limiting the efficiency of chitin degradation. Chitin polymerisation is similar to cellulose polymerisation, and the adjacent polymer chains contain abundant hydrogen bonds forming various ordered crystalline arrangements. In this context, the expansin-like proteins CxEXL22 and HcEXL act on the hydrogen bonds between the chitin polysaccharide chains to split them and disrupt the orderly lattice arrangement of chitin, causing significant depolymerisation and exposure of more microfibrils, which increases the available surface area and thus, enhances chitin accessibility. After pretreatment, the crystallinity index of the chitin decreased, and both the d-spacing and crystallite size increased. This indicates that the hydrogen

bonds between the hydroxy groups of the chitin chains were weakened, causing the pretreated chitin to swell. The d-spacing of the pretreated chitin increased slightly owing to swelling, leading to an increase in crystallite size. Thus expansin-like proteins exhibited synergistic catalytic activity on chitin with chitinase to improve chitin degradation. These results indicate that the expansin-like proteins CxEXL22 and HcEXL for pretreating chitin is a novel and promising method for reducing the environmental burdens in chitin conversion.

Funding

This work was greatly acknowledged by the National Natural Science Foundation of China (No. 31600640), Liaoning province education sciences foundation (LJKMZ20220886, LJKQZ2021114), Dalian Key Research and Development Program Project of Dalian (2022YF16SN064) and Science and technology Department of Dalian (2020RQ118) for financial support.

Ethics declarations

Not applicable.

Data availability statement

All available data are presented within the article.

CRediT authorship contribution statement

Rong Li: Writing – original draft, Supervision, Funding acquisition, Conceptualization. **Chang Jiang:** Data curation. **Xiaodan Li:** Validation. **Yihao Zhou:** Data curation. **Linlu You:** Validation. **Qiushi Wang:** Writing – review & editing. **Wenzhu Tang:** Funding acquisition, Conceptualization. **Zhimin Yu:** Funding acquisition, Conceptualization. **Fan Yang:** Supervision, Funding acquisition. **Xianzhen Li:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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