Efficient Degradation of Alginate And Preparation of Unsaturated Monosaccharides By A Novel Alginate Lyase And Effects of Domain Truncation On Biochemical Characteristics, Degradation Patterns

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Research

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Abstract

Background

Brown algae are considered promising crops for the production of sustainable biofuels. However, its commercial application has been limited by lack of efficient methods for converting alginate into fermentable sugars. Recently, exo-type alginate lyases have received extensive attention due to their excellent ability of conversion of alginate into 4-deoxy-L-erythro-5-hexoseulose uronate (DEH), a promising material for bioethanol production and biorefinery systems.

Results

Herein, we cloned and characterized a novel alginate lyase AlyPL17 from *Pedobacter hainanensis* NJ-02. It possessed outstanding catalytic efficiency towards polymannuronic acid (polyM), polyguluronic acid (polyG) and alginate sodium, with k_{cat} of $39.42 \pm 1.9 \text{ s}^{-1}$, $32.53 \pm 0.88 \text{ s}^{-1}$, and $38.30 \pm 2.12 \text{ s}^{-1}$, respectively. In addition, AlyPL17 adopts a unique hybrid action mode to degrade alginate by the synergistic effect of two domains. Furthermore, the combination of AlyPL17 and AlyPL6 exhibited apparently synergistic effect for the preparation of unsaturated monosaccharides.

Conclusion

Overall, the results show that AlyPL17 is a PL17 exo-type alginate lyase with high activity and a high conversion rate at low/moderate temperatures, which provides a useful enzymatic tool for the conversion of brown algae into biofuels and enhance our understanding of the function of modular domain of alginate lyase.

Background

With the global demand for renewable energy production increased markedly, methods for the production of bioethanol have been significantly improved in the past decade (Wargacki et al., 2012). Corn, sugarcane and several inedible lignocellulosic materials are preferable feedstocks (Takeda et al., 2011). However, it is difficult to convert the inedible lignocellulosic materials into unsaturated monosaccharides. In addition, using edible biomass could lead to bioethanol-food conflicts. Last but not least, large-scale cultivation of biofuel crops may lead to a net increase in greenhouse gases (Takeda et al., 2011). Requiring no arable land, and fresh water resources, marine algae has been considered as the third-generation feedstocks (Takeda et al., 2011).

The most abundant sugars in brown alga are alginate, mannitol and glucan (such as cellulose and laminarin) (Enquist-Newman et al., 2014). In recent years, many researchers have developed various strategies for fermenting these sugars into bioethanol (Wargacki et al., 2012). However, the utilization of alginate for bioethanol production is still a major bottleneck. Alginate, an acidic polysaccharide, consists of β -D-mannuronic acid (M) and α -L-guluronic acid (G) (Zhu & Yin, 2015). Several researchers have developed metabolic engineered microorganisms for fermenting alginate into ethanol. For example, Takeda *et al.* constructed a metabolically modified bacterium, *Sphingomonas* sp. A1, which could accumulate ethanol using alginate as the sole carbon source (Takeda et al., 2011). The metabolic pathway of alginate is as follows: firstly, endolytic alginate lyase could cleave the glycosidic bonds to create alginate oligosaccharides with various degrees of polymerization (Li et al., 2020), while exolytic lyase could release unsaturated monosaccharides or disaccharides as products (Tøndervik et al., 2010). Unsaturated monosaccharides could be converted into 4-deoxy-L-erythron-5-hexoseuloseuronate acid (DEH) through spontaneous conversion or KdgF catalysis (Hobbsa et al., 2017). Then DEH can be reduced to 2-keto-3-dexoxy-D-gluconate (KDG) before entering the Entner-Doudoroff (ED) pathway to produce two molecules of pyruvate, which may converted to two molecules of ethanol (Hobbsa et al., 2017) (Fig. 1). Thus, the degradation of alginate into unsaturated monosaccharides is an essential step for the preparation of bioethanol from alginate.

Various exolytic alginate lyases from several polysaccharide lyase families have been cloned and characterized in recent years. For instance, AlyGC (PL6 family) from *Glaciecola chathamensis* S18K6^T (Xu et al., 2017), VxAly7D (PL7 family) from *Vibrio xiamenensis* QY104 (Tang et al., 2020), Alg17c (PL17 family) from *Saccharophagus degradans* 2-40 (Kim et al., 2012) and Atu3025 (PL15 family) from *Agrobacterium tumefaciens* (Ochiai et al., 2010). Nevertheless, most exolytic alginate lyases have low activity, resulting in low unsaturated monosaccharide yield. In addition, almost all of the alginate metabolic bacteria contain two or three putative alginate lyases with different action mode and substrate specificity (Li et al., 2018). However, complementary or synergistic effects of those alginate lyases have been rarely evaluated (Li et al., 2018). Thus, evaluating synergistic effects of the alginate lyase from the same bacteria, and establishing an efficient method for the production of unsaturated monosaccharides are urgent problems to be solved.

In our previous work, we cloned and expressed a novel endolytic alginate lyase AlyPL6 from *Pedobacterhainanensis* NJ-02, which could degrade alginate into alginate oligosaccharides with high efficiency (Li et al., 2020). Herein, we cloned and characterized a new PL17 family alginate lyase AlyPL17 from the same bacteria. The biochemical characterization and action pattern of AlyPL17 were investigated. Additionally, we initially explore the mechanism of action mode of this enzyme through domain truncation. We hypothesized that AlyPL6 and AlyPL17 exhibit better synergistic effects, and can degrade alginate completely. This study is expected to provide efficient enzyme for the degradation of alginate and production of biofuels.

Results And Discussion

Sequence Analysis

The open reading frame (ORF) of AlyPL17 consists of 2085bp encoded a putative alginate lyase composed of 695 amino acids with a theoretical molecular mass of 78.07 kDa. As shown in Fig. 2A, the full-length enzyme contains two domains, an alginate lyase domain (designed as AlyPL17-N, Lys₃₇-Gln₂₆₅) and a heparinase II/III-like domain (designed as AlyPL17-C, Arg₃₅₇-Phe₅₂₈). The phylogenetic tree was constructed and it exhibited AlyPL17 clusters with several alginate lyases of PL17 family. Based on the alignment of protein sequence, AlyPL17 has the highest sequence identity (45%) with OAL from *Stenotrophomonas maltophilia* KJ-2 (GenBank accession no. AGM38186.2) (Shin et al., 2015), followed by Alg17c (44%) from *Saccharophagus degradans* 2–40 (GenBank accession no. ABD82539.1) (Park et al., 2014). Furthermore, several key amino acid residues of PL17 family are highly conserved in AlyPL17 such as N₁₇₇, H₁₇₈, Y₂₃₄, Y₄₂₆ in active sites; N₁₂₅, R₂₃₆, Y₂₃₇, H₃₈₉, R₄₁₄ in the substrate binding sites.

Based on the homologous structure of Alg17c (PDB: 4NEI) from *Saccharophagus degradans* 2–40, three-dimensional models of AlyPL17 and its truncated mutants were constructed by PHYRE2. The sequence identity between AlyPL17 and Alg17c was high (44%). Accordingly, the protein model was successfully constructed with 100% confidence. As shown in Fig. 2B, AlyPL17 harbors two domains, AlyPL17-N is folded as a (α/α)₆ toroid fold, and AlyPL17-C is folded as a β -sandwich jellyroll with two anti-parallel β sheets (Fig. 2C, 2D).

Expression and Purification of AlyPL17 and Its Truncated Mutants

After heterologously expressed and purified, the recombinant AlyPL17, AlyPL17-N and AlyPL17-C were obtained and then analyzed by SDS-PAGE. In Fig. 3, three clear bands represented the molecular mass of purified AlyPL17, AlyPL17-N and AlyPL17-C can be observed, which are consistent with the predicted molecular mass of 78.07 kDa, 39.22 kDa and 39.78 kDa, respectively. The molecular mass (Mr) of other PL17 alginate lyases varied from 75 kDa to 86 kDa. For example, OalB from *Vibrio splendidus* 12B01 (Jagtap et al., 2014), Alg17c from *Saccharophagus degradans* 2–40 (Park et al., 2014) and Oal17A from *Vibrio* sp. W13 (Lyu et al., 2018) possess the molecular mass of 83 kDa, 79 kDa and 81 kDa, respectively.

Three different substrates (0.5% sodium alginate, 0.5% polyM and 0.5% polyG) were applied to determine the activities of AlyPL17 and its truncated mutants. As shown in Table 1, the full-length enzyme AlyPL17 exhibited higher activities towards polyM (861.62 ± 1.53 U/mg) than towards sodium alginate (808.53 ± 2.23 U/mg), and polyG (802.62 ± 2.45 U/mg). To the best of our knowledge, AlyPL17 has relatively higher enzymatic activities than most PL17 family alginate lyases. For instance, the activities of AlyPL17 towards sodium alginate, polyM and polyG are 2.0, 1.71 and 3.97 times higher than the activities of Alg17B (MacDonald et al., 2016). Moreover, the activities of AlgSH17 towards sodium alginate, polyM and polyG were 55.62 U/mg, 116.8 U/mg, 30.28 U/mg, respectively, which are 14.53, 7.73 and 26.50 times lower than that of AlyPL17 (Yang et al., 2021). Accordingly, AlyPL17 was an excellent tool for the degradation of alginate. In contrast, two truncated mutants (AlyPL17-N and AlyPL17-C) maintained only 3–5% of its initial activity. It indicates that both AlyPL17-N and AlyPL17-C domains were essential for maintaining activity. Furthermore, the K_m values of AlyPL17 with polyM, sodium alginate and polyG as substrates were 3.35 ± 0.62 , 3.62 ± 0.23 and 4.89 ± 0.71 mM, respectively. Thus, AlyPL17 was considered to prefer polyM block region in alginate polymer. Additionally, the k_{cat} values of AlyPL17 towards polyM, sodium alginate and polyG were 39.42 ± 1.69 , 38.30 ± 2.12 and 32.53 ± 0.88 s⁻¹, respectively. It suggests that AlyPL17 was a novel alginate lyase specific for polyM. Similarly, most alginate lyases from the PL17 family were polyM-preferred lyases, such as OalC17 from *Cellulophaga* sp. SY116 (Wang et al., 2014), AlgL17 from *Microbulbifer* sp. ALW1 (Jiang et al., 2019), and Smlt2602 from *Stenotrophomonas maltophilia* K279a (MacDonald et al., 2016).

Table 1
substrate specificity and kinetics of AlyPL17

Substrate	Sodium alginate	PolyM	PolyG
Activity (U/mg)	808.53 ± 4.23	861.62 ± 3.53	802.62 ± 3.45
K_m (mM)	3.62 ± 0.23	3.35 ± 0.62	4.89 ± 0.71
V_{max} (μmol/s)	9.96 ± 0.95	10.24 ± 0.68	8.45 ± 1.02
k_{cat} (s ⁻¹)	38.30 ± 2.12	39.42 ± 1.69	32.53 ± 0.88
k_{cat}/K_m (s ⁻¹ /mM)	10.58 ± 2.15	11.76 ± 2.36	6.65 ± 0.77

Biochemical Characterization of AlyPL17 and Its Truncated Mutants

For biochemical characterization of AlyPL17, AlyPL17-N and AlyPL17-C, both AlyPL17 and its truncated mutants exhibited the highest activities at 45 °C (Fig. 4A) and pH 9.0 (Fig. 4D). As shown in Table 2, the optimal reaction conditions of alginate lyases from PL17 family are in the range of 30 to 50°C, and pH 6.0 to 8.0. For example, OalS17 (Wang et al., 2014) from *Shewanella* sp. Kz7 showed the highest activity at 50°C and pH 6.2, and TcAlg1 from *Thalassotalea crassostreae* exhibited the maximal activity at 40°C and pH 7.0 (Wang et al., 2018). Moreover, the optimal temperature and pH of Oalv17 were 40°C and 7.2 (Li et al., 2020), respectively. In terms of temperature stability, AlyPL17 retains about 40% activity after being incubated at 40°C for 1 h (Fig. 4B), which was better than several PL17 family alginate lyases. For instance, OalC17 retains 20% of maximal activity after being incubated at 40°C for 1 h (Li et al., 2018), and AlgSH17 could only retain stability at 20-25°C (Yang et al., 2021). Interestingly, the two truncated mutants exhibited excellent thermal stability, AlyPL17-N and AlyPL17-C could retain about 95% and 80% of its maximal activities after being incubated at 40°C for 1 h (Fig. 4C). This phenomenon was also shown in truncated mutant Aly7B-CDII, which exhibited greater temperature stability than full-length enzyme Aly7B at 35°C (Hu et al., 2019). Similarly, the thermal ability of truncated AlgH-I and AlgH-II are 2.1 and 1.3-fold higher than full-length enzyme AlgH (Yan et al., 2019). Perhaps the truncated mutants have a more compact structure than full-length enzyme and thereby resist the effects of thermal degeneration (Hu et al., 2019). AlyPL17 reserves more than 60% of its maximal activities after being incubated at pH 6.0–9.0 for 24 h. Whereas, AlyPL17-N and AlyPL17-C maintained better stability at pH 9.0 (Fig. 4E). In further characterization, we introduced various metal ions to explore the effects on enzymatic activity. As shown in Fig. 4F, AlyPL17 was activated by Mg²⁺ and Mn²⁺. Moreover, AlyPL17-N could be activated by Na⁺. In addition, Co²⁺, Zn²⁺, Cu²⁺ and Fe³⁺ can inhibit the activities of AlyPL17 and its truncated mutants, as known for many other alginate lyases from PL17 Family.

Table 2 Characteristics of alginate lyases from PL17 family

Enzyme	Action mode	Optimal temperature/pH	Temperature/pH stability	Substrate preference	Source	Reference
AlyPL17	endo+exo	45°C/9.0	35°C, 60 min, remained 97%; pH 6.0-9.0, 24h, remained 60%	polyM	<i>Pedobacter hainanensis</i> NJ-02	This study
Oal17A	exo	30°C/7.0	45°C, pH 6.0-9.0, remained ≥ 80%	polyMG	<i>Vibrio</i> sp. W13	(Lyu et al., 2018)
OalV17	exo	40°C/7.2	40°C, 60 min, remained 74.5%; pH 3.4-8.0, 12h, remained 70%	polyM	<i>Vibrio</i> sp. SY01	(Li et al., 2020)
OalB	exo	30°C/7.0	NA	polyMG	<i>Vibrio splendidus</i> 12B01	(Jagtap et al., 2014)
OalC	exo	35°C/7.5	NA	polyM	<i>Vibrio splendidus</i> 12B01	(Jagtap et al., 2014)
OAL	endo+exo	37°C/7.5	NA	polyM	<i>Stenotrophomonas maltophilia</i> KJ-2	(Shin et al., 2015)
Alg17C	exo	40°C/6.0	NA	polyM, polyG	<i>Saccharophagus degradans</i> 2-40	(Park et al., 2014)
Alg17B	endo+exo	45°C/7.5-8.0	40°C, 60 min, remained 40%; pH 6.5-8.0, 24h, remained 75%	Bi-	<i>Vibrio</i> sp. BP-2	(Huang et al., 2019)
FIAllyB	exo	NA	NA	polyM	<i>Flavobacterium</i> sp. strain UMI-01	(Nishiyama et al., 2017)
TcAlg1	exo	40°C/7.0	40°C, 60 min, remain unchanged	polyM	<i>Thalassotalea crassostreae</i>	(Wang et al., 2018)
OalS17	exo	50°C/6.2	40°C, 60 min, remained 88%; pH 6.0-9.0, 6h, remained stable	polyMG	<i>Shewanella</i> sp. Kz7	(Wang et al., 2014)
AlgL	endo+exo	50°C/6.5	NA	polyM	<i>Sphingomonas</i> sp. MJ-3	(Jiang et al., 2019)
OalC17	exo	50°C/7.2	40°C, 60 min, remained 20%; pH 6.0-8.0, 6h, remained 85%	polyM	<i>Cellulophaga</i> sp. SY116	(Li et al., 2018)
SmIt2602	exo	-/8.5	NA	polyM	<i>Stenotrophomonas maltophilia</i> K279a	(MacDonald et al., 2016)
AlgSH17	endo+exo	30°C/7.0	40°C, 60 min, remained less than 20%; pH 6.0-8.0, 2h, remained 80%	polyM	<i>Microbulbifer</i> sp. SH- 1	(Yang et al., 2021)

Analysis of Action Pattern and Degradation Products of AlyPL17 and Its Truncated Mutants

Degradation products of AlyPL17 and its truncated mutants towards sodium alginate at various times (0–48 h) were analyzed by Fast protein liquid chromatography (FPLC). As shown in Fig. 5A, at preliminary stage of reaction, AlyPL17 could degrade alginate into dimers and monosaccharides. However, dimers were degraded into monosaccharides after incubation for 48 h. In accordance with the results above, AlyPL17 adopts a unique action mode that combines the endolytic action pattern and exolytic action pattern. ESI-MS was used to analyze the composition of the end products (Fig. 6A), one molecular ion peak at 175.02 m/z $[\Delta\text{DP1-H}]^-$ was individually detected in the corresponding fraction. As shown in Table 2, although most alginate lyases from the PL17 family adopt exolytic mode to degrade alginate such as OalC17 from *Cellulophaga* sp. SY116 (Li et al., 2018) and FIAlyB from *Flavobacterium* sp. strain UMI-01 (Nishiyama et al., 2017). This unique hybrid action pattern also has been adopted by several alginate lyases of PL17 family. For example, Alg17B from BP-2 (new species) could degrade alginate into alginate oligosaccharides with DP1-6 at preliminary stage of reaction, and then degrade alginate oligosaccharides into disaccharides and monosaccharides (Huang et al., 2019). Moreover, in the early stages of the reaction, OAL from *Stenotrophomonas maltophilia* KJ-2 could produce alginate oligosaccharides with DP 1–4, and gradually degrade oligosaccharide into monosaccharide. Furthermore, AlgL from *Sphingomonas* sp. MJ-3 degrades alginate into alginate oligosaccharides with DP2-4 in endolytic action mode, and then it could degrade alginate oligosaccharides into monosaccharide in exolytic action mode (Jiang et al., 2019). Additionally, AlgSH17 from *Microbulbifer* sp. SH-1 also possesses both endolytic and exolytic activity (Yang et al., 2021). To explore the functions of the two domains, we analyzed the products distribution of AlyPL17-N and AlyPL17-C. As shown in Fig. 5B, AlyPL17-N exhibited a typical endolytic action pattern, which could degrade alginate into dimers, trimers, and tetramers. The final products had three molecular ion peaks at 268.79 m/z $[\text{DP3} + \text{H}_2\text{O-H}]^{2-}$, 351.72 m/z $[\text{DP2-H}]^-$, 657.98 m/z $[\text{DP4} + 4\text{H-3Na}]^-$ (Fig. 6B). In addition, AlyPL17-C also showed endolytic degradation mode towards sodium alginate (Fig. 5C), and the main products of AlyPL17-C were oligosaccharide with DP1-4 (Fig. 6C). The final products had four molecular ion peaks at 160.84 m/z $[\Delta\text{-H}_2\text{O} + 3\text{H}]^{2-}$, 268.79 m/z $[\text{DP3} + \text{H}_2\text{O-H}]^{2-}$, 351.72 m/z $[\text{DP2-H}]^-$, 657.98 m/z $[\text{DP4} + 4\text{H-3Na}]^-$ (Fig. 6C). Similarly, the two truncated mutants of OAL adopt endolytic action mode to degrade alginate, and the main products of the mutants are DP1-6 (Shin et al., 2015). The hybrid activity of AlyPL17 perhaps is caused by the synergistic effects of AlyPL17-N and AlyPL17-C.

Synergistic Effect of AlyPL17 and AlyPL6

Although AlyPL17 exhibited relatively higher activities towards alginate than most of alginate lyases from PL17 family, it still showed lower catalysis efficiency than most endolytic alginate lyases. In our previous work, we cloned and characterized a novel endolytic alginate lyase AlyPL6 with high activity from *Pedobacter hainanensis* NJ-02 (Li et al., 2020). Since AlyPL17 has the ability to degrade alginate oligosaccharides into monosaccharide, it may prefer digesting the oligosaccharides with low DPs. Additionally, AlyPL17 and AlyPL6 were isolated from the same strain and have similar optimal reaction conditions. Last but not least, AlyPL17 and AlyPL6 have the complementary substrate specificity, AlyPL6 was polyMG preferred alginate lyase, and AlyPL17 exhibited the highest activities towards polyM. Therefore, AlyPL6 and AlyPL17 may exhibit a synergistic degradation effect towards alginate. To investigate this possibility, the enzymatic assay was applied to evaluate the catalytic ability of AlyPL6 plus AlyPL17 (AlyPL6/AlyPL17 ratio: 0, 1:9, 2:8, 3:7, 4:6, and 5:5).

As shown in Supplementary Table S1, as the percentage of AlyPL6 goes up, the activities towards alginate have been improved obviously. When the ratio of AlyPL6/AlyPL17 reaches 4:6, the degradation activity towards alginate reached the maximum. Furthermore, in order to compare the monosaccharide yield of AlyPL6 plus AlyPL17 (AlyPL6/AlyPL17 ratio: 4:6) and AlyPL17 alone, FPLC was used for products analysis. As shown in Fig. 7A, a total of $10\mu\text{g}$ AlyPL6 plus AlyPL17 ($4\mu\text{g}$ AlyPL6 and $6\mu\text{g}$ AlyPL17) could convert 10 mg of alginate into unsaturated monosaccharide with a yield of 21.4% after being incubated 10 min. In addition, the monosaccharides yield of AlyPL6 plus AlyPL17 (45%) was 1.96 times higher than AlyPL17 (23%) after being incubated at 45°C for 1 h (Fig. 7B). Similarly, Li et al. established the method for the production of monosaccharide by combining endolytic alginate lyase AlySY08 and exolytic alginate lyases (OalC6 and OalC17) (Li et al., 2018). However, the activities and the temperature stabilities of OalC6 and OalC17 are weaker than AlyPL6 and AlyPL17 (Li et al., 2018). Accordingly, the combination of AlyPL6 and AlyPL17 is an efficient and stable tool for the production of biofuels from alginate.

Conclusion

In conclusion, a novel PL17 family AlyPL17 from *Pedobacter hainanensis* NJ-02 was cloned and expressed. Its biochemical properties and action pattern were clarified. Furthermore, to investigate the function of the two domains, the N-terminal domain (AlyPL17-N) and C-terminal (AlyPL17-C) domain of AlyPL17 were subcloned and expressed, independently. AlyPL17 shared the highest identity of 45% with

OAL and showed optimal activity at 45°C and pH 9.0. Compared with other alginate lyases from the PL17 family, AlyPL17 has relatively high activity. Nevertheless, two truncated mutants AlyPL17-N and AlyPL17-C showed decreased activities, which indicated that both AlyPL17-N and AlyPL17-C domains are essential to maintain high activity of the full-length enzyme. AlyPL17 could degrade alginate into monosaccharide by a hybrid action mode that combined the endolytic action pattern and exolytic action pattern. However, AlyPL17-N and AlyPL17-C exhibited an endolytic action pattern towards alginate. It suggested that AlyPL17-N and AlyPL17-C might show a synergistic effect, resulting in high exolytic activity of full-length enzyme AlyPL17. Last but not least, AlyPL6 and AlyPL17 exhibit excellent synergistic effects. When AlyPL6 and AlyPL17 were combined in a ratio of 4:6, the unsaturated monosaccharide conversion rate reached 45% in one hour. This work provides efficient enzymes for the development of biofuels from marine brown algae.

Materials And Methods

Materials and Strains.

Sodium alginate from *Macrosystis pyrifera* was purchased from Sigma-Aldrich (M/G ratio 77/23, viscosity ≥ 2000 Cp, St. Louis, MO, USA). PolyM and polyG (purity: about 95%, M/G ratio: 97/3 and 3/97, respectively) were purchased from Qingdao BZ Oligo Biotech Co., Ltd. (Qingdao, China). *Pedobacter hainanensis* NJ-02(CCTCC M2016579) was isolated from the East China Sea and conserved in our laboratory. *E. coli* BL21 (DE3) was used for gene expression and was grown in Luria–Bertani (LB) broth or on LB broth agar plates (LB broth supplemented with 1.0% agar) including 100 mg/mL ampicillin.

Sequence Analysis

The conserved domains of AlyPL17 were predicted using the NCBI database by the CD-search application (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). The vector-NTI (Life Technologies, Grand Island, NY) was used for homology analysis of protein sequence. The phylogenetic tree was constructed according to the relative protein sequences of PL17 family through molecular evolutionary genetics analysis (MEGA) program version 6.0. According to the structure of alginate lyase Alg17c from *Saccharophagus degradans* 2-40 (PDB: 4NEI), the three-dimensional structure of AlyPL17, AlyPL17N and AlyPL17C were constructed through Homology/analogY Recognition Engine V 2.0. The final structures of AlyPL17 and its truncated mutants were viewed in the PyMOL software.

Heterologous Expression and Purification of Recombinant Alginate Lyase

The genes of AlyPL17, AlyPL17-N and AlyPL17-C were synthesized by GENEWIZ (Suzhou, Jiangsu province, China). These three genes were ligated into pET-21a (+) expression vector and were then transformed into *E. coli* BL21 (DE3). All recombinant strains were cultured and induced as previously reported (Zhu et al., 2019a). After culturing cells at 22°C for 36h, the AlyPL17, AlyPL17-N and AlyPL17-C were purified by Ni-NTA sepharose column (GE Healthcare, Uppsala, Sweden) and HiTrap™ desalting column (Amersham Biosciences, Buckinghamshire, UK) (Hu et al., 2019). Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was applied to analyze the purity of the recombinant proteins. Furthermore, the protein quantitative analysis kit (Beyotime Institute of Biotechnology, Nantong, China) was used to determine the concentration of recombinant proteins.

Substrate Specificity and Enzymatic Kinetics

The activities of AlyPL17 and its two truncated mutants were investigated using the ultraviolet absorption method described by Inoue (Zhu et al., 2019). 20 mL purified recombinant proteins were incubated with 180 mL 0.5% (w/v) substrates (sodium alginate, polyM and polyG dissolved in 50 mM glycine-NaOH buffer pH 9.0) at 45°C for 10 min. One-unit enzymatic activity was defined as the amount of enzyme required to increase the absorbance at 235 nm (extinction coefficient: $6150 \text{ M}^{-1} \cdot \text{cm}^{-1}$) by 0.1 per min. The kinetic parameters of AlyPL17 towards different substrates were studied through measuring activities with substrates at different concentrations (0.4–4 mg/mL). Velocity (V) at different substrate concentrations was calculated as follows: $V (\text{mol/s}) = (\text{milliAU/min} \times \text{min}/60 \text{ s} \times \text{AU}/1000 \text{ milliAU} \times 1 \text{ cm}) / (6150 \text{ M}^{-1} \text{ cm}^{-1}) \times (2 \times 10^{-4} \text{ L})$ (Zhu et al., 2019). The K_m , V_{max} values of AlyPL17 were calculated by hyperbolic regression analysis as previously reported (Li et al., 2020). The ratio of V_{max} versus enzyme concentration ($[E]$) was used to calculate the turnover number (k_{cat}) of the enzyme.

Biochemical Characterization of AlyPL17 and Its Truncated Mutants

To determine the optimal temperature and the thermal stability of AlyPL17, AlyPL17-N and AlyPL17-C, reactions were carried out at 30°C–55°C. Additionally, the thermally induced denaturation was also investigated by evaluating the residual activities after incubating at 40–45°C for 0–60 min. The influence of pH on enzymatic activities of these enzymes was studied by measuring the activities in presence of different buffers (50 mM phosphate-citrate (pH 4.0–5.0), 50 mM NaH₂PO₄-Na₂HPO₄ (pH 6.0–8.0), 50 mM Tris-HCl (pH 7.0–9.0) and 50 mM glycine-NaOH (pH 9.0–10.0)). Moreover, the pH stability was determined through assessing the residual activities after being incubated in different pH buffers at 4°C for 24 h. Furthermore, to investigate the effects of metal ions on enzymatic activities, purified enzymes were incubated with various metal ions (1 mM) at 4°C for 4 h. The reaction mixture without any metal ions was regarded as control (100% relative activity). In order to ensure the accuracy of the experiments, all experiments were performed with three replicates.

Action Pattern and Degradation Products Analysis

In order to explore the action pattern and degradation products of AlyPL17 and its truncated mutants, 50 mL purified enzymes (10 mg) were incubated with 450 mL 0.5% sodium alginate at 40°C for 0–48 h. The samples were taken after reaction for 1, 8, 24, 48 h, respectively. Fast protein liquid chromatography equipped with superdex peptide 10/300 GE Column (GE Health) was applied to analyze the degradation products of AlyPL17 and its two truncated mutants as previously reported (Li et al., 2020). In addition, the end products of AlyPL17 and its truncated mutants were analyzed by ESI-MS in a positive-ion mode using the following settings: ion source voltage, 4.5 kV; capillary temperature, 275–300 °C; Tube lens, 250 V; sheath gas, 30 arbitrary units (AU); scanning mass range, 150–2000 *m/z*.

Analysis of the Synergistic Effect of AlyPL17 and AlyPL6

To investigate the optimal synergistic degradation ratio, 20 mL AlyPL6 plus AlyPL17 (0.2 mg/mL, AlyPL6/AlyPL17 ratio: 0, 1:9, 2:8, 3:7, 4:6, and 5:5) were added to 180 mL alginate (5 mg/mL in 50 mM glycine-NaOH buffer pH 9.0) and incubated at 45 °C for 10 min. Ultraviolet absorption method was applied to measure the activity of AlyPL6 plus AlyPL17. Furthermore, the composition of end products of optimal synergistic degradation ratio were analyzed by FPLC as previously reported (Li et al., 2020).

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

QL and SSC done the experiment and collected the data, QL, SSC and ZL wrote the manuscript, and ZBW revised this work. All authors read and approved the manuscript.

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Availability of data and material

Not applicable.

Supporting information

Nucleotide sequence of AlyPL17 and the parameter of synergistic reaction of AlyPL6 and AlyPL17 as deposited additional files.

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Figures

Figure 1

The schematic diagram of alginate metabolism. DEH represents 4-Dexoy -L- erythron -5-hexoseulose Uronate.

Figure 2

Modular organization of alginate lyase AlyPL17. (A) Module organization of AlyPL17. It contains an alginate lyase-like domain (AlyPL17-N, Lys37-Gln265) and a Heparinase-like domain (AlyPL17-C, Arg357-Phe528); (B) The model structure of AlyPL17; (C) The model structure of AlyPL17N; (D) The model structure of AlyPL17C.

Figure 3

SDS-PAGE analysis of purified AlyPL17, AlyPL17-N and AlyPL17-C. Lane M, molecular weight markers; Lane 1, purified AlyPL17; Lane 2, purified AlyPL17-N; Lane 3, purified AlyPL17-C

Figure 4

Biochemical characterization of AlyPL17 and its two truncated mutants. (A) The optimal temperature of AlyPL17 and its two truncated mutants. (B) The thermal stability of AlyPL17 and its two truncated mutants. (C) The thermal-induced denaturation of AlyPL17 and its two truncated mutants at 40°C and 45°C. (D) The effect of pH on the enzymatic activity of AlyPL17 and its two truncated mutants. (E) The pH stability of AlyPL17 and its two truncated mutants. (F) The influence of metal ions of AlyPL17 and its two truncated mutants.

Figure 5

FPLC analysis of the degradation products for 48 h. (A) Degradation products generated by AlyPL17 (B) Degradation products generated by AlyPL17-N, (C) Degradation products generated by AlyPL17-C. The eluents were detected by measuring the absorbance at 235 nm. The elution volumes of the unsaturated monomer, dimer, trimer, and tetramer are 17.35, 16.25, 15.23, and 14.38mL.

Figure 6

ESI-MS analysis for the degradation products of (A) AlyPL17, (B) AlyPL17-N, and (C) AlyPL17-C for 48 h.

Figure 7

Analysis of the synergistic effect by FPLC. (A) Alginate was treated with a mixture (10 mg) of 6µg AlyPL17 and 4µg AlyPL6 at 45oC for 10 min. By contrast, control was treated with only 10 µg AlyPL6 or only 10 µg AlyPL17 at the same conditions. (B) Alginate was treated with mixture, AlyPL6, or AlyPL17 at 45oC for 1 h.

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