NOTE / NOTE

Cloning, sequence analysis, and expression of gene *alyPI* encoding an alginate lyase from marine bacterium *Pseudoalteromonas* sp. CY24

Gaofei Duan, Feng Han, and Wengong Yu

Abstract: The alginate lyase encoding gene (*alyPI*) of marine bacterium *Pseudoalteromonas* sp. CY24 was cloned using a battery of PCR techniques. Gene *alyPI* was composed of a 1575 bp open reading frame encoding a protein of 57.4 kDa containing 524 amino acid residues with a signal peptide of 23 amino acids. The AlyPI protein was expressed in *Escherichia coli* with a His-tag sequence fused at the C-terminal end and purified to electrophoretic homogeneity using Ni-sepharose affinity chromatography. AlyPI was most active at 40 °C and pH 7.0 in the presencce of 0.1 mol/L NaCl and stable over a broad range of pH, 6.0–10.6. The presence of Na^+, K^+, Mn^{2+}, Ca^{2+}, and Fe^{3+} can enhance the enzyme activity. The alginate lyase consensus region YFKAGXYXQ, regarded as a striking feature at the C termini of several alginate lyase of ~30 kDa, was found in AlyPI, which belongs to the ~60 kDa group. Another nine amino acid consensus region, YXRSELREM, only found in G-specific alginate lyases previously existed in AlyPI, which could degrade sodium alginate, M blocks, and G blocks and appeared to be a broad substrate-specific alginate lyase.

Key words: alginate lyase, cloning, expression, *Pseudoalteromonas*.

Résumé : Le gène qui code l'alginate lyase (*alyPI*) chez la bactérie marine *Pseudoalteromonas* sp. CYP24 a été cloné à l'aide d'une batterie de techniques de PCR. Le gène *alyPI* était composé d'un cadre de lecture ouvert de 1 575 pb codant une protéine de 57.4 kDa qui contient 524 acides aminés dont un peptide signal de 23 acides aminés. La protéine AlyPI contenant une séquence His-tag fusionnée à l'extrémité C-terminale a été exprimée chez *Escherichia coli* et purifiée à homogénéité électrophorétique par chromatographie d'affinité sur Ni-sepharose. AlyPI était plus active à 40 °C et à pH 7.0 en présence de 0,1 mol/L NaCl et était stable dans un large éventail de pH de 6,0 à 10,6. La présence de Na^+, K^+, Mn^{2+}, Ca^{2+} et Fe^{3+} peut augmenter l'activité de l'enzyme. La région consensus YFKAGXYXQ de l'alginate lyase, considérée comme une caractéristique remarquable de l'extrémité C-terminale de plusieurs alginate lyases de ~30 kDa, a été trouvée chez AlyPI, qui appartient au groupe de ~60 kDa. Une autre région consensus de neuf acides aminés, YXRSELREM, trouvée uniquement chez les alginate lyases G-spécifiques existait chez AlyPI, laquelle pouvait dégrader l'alginate de sodium, les blocs M et G, et qui semblait être une alginate lyase possédant une spécificité de substrat étendue.

Mots-clés : alginate lyase, clonage, expression, *Pseudoalteromonas*.

[Traduit par la Rédaction]

Alginate is a linear heteropolyuronic acid polysaccharides in which β-D-mannuronic acid (M) and its C5 epimer α-L-guluronic acid (G) are (1,4)-linked to form blocks of consecutive G residues (G blocks), consecutive M residues (M blocks), and alternating M and G residues (MG blocks) (Gacesa 1988). Alginate is synthesized as part of the cell wall and intracellular material by brown seaweeds and as exopolysaccharides by some heterotropic bacteria belonging to the genera *Azotobacter* and *Pseudomonas* (Govan et al. 1981; Wong et al. 2000). Alginate lyases catalyze the depolymerization of alginates through β-elimination of the 4-*O*-glycosyl bond accompanied by the formation of a double bond between C4 and C5 and the production of 4-deoxy-L-erythro-hex-4-enopyranosyluronic acid at the nonreducing end of the resulting oligosaccharides (Gacesa 1992). On the basis of their substrate specificities, alginate lyases are classified as polymannuronate lyase (EC 4.2.2.3) and polyguluronate lyase (EC 4.2.2.11). According to their molecular mass, most alginate lyases appear to fall into three major classes: 20–35, ~40, and ~60 kDa. The best alignments were found in the ~40 kDa group by comparing the primary sequence alignments for alginate lyases in each size group (Wong et al. 2000).

Since the original description of alginate lyases, more than 50 enzymes have been characterized from various sour-

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Fig. 1. Nucleotide sequence and deduced amino acid sequence of the gene *alyPI* encoding alginate lyase. Nucleotide and amino acid numbering (bold) is shown on the right. Other data include the start and stop codons (bold), the putative –35 and –10 sequences for the promoter and the possible ribosome-binding site (solid underline), the putative terminator sequences (a pair of inverted arrows), and the possible signal peptide (broken underline).

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AGATCTTAATCGCATTTATCAACATTCATATATATTTGTATTATTAAATTATATTATCTT   60
TGTGGTTCATTAGATTTGAAAACGCTCTCGTATAGGGTTTCAGATAATGTGGAAACGGAG  120
                -35                      -10
ATAGCTGTTATTTCCGCGTCAACACACAGCACTGAGTCGATTATATAAAAAACTATATAG  180
TTGATTTATACAACATTAGGTGAATGGATACATGAAACATATCTATCTTAAAAGCTTGTT  240
            RBS          M  K  H  I  Y  L  K  S  L  L          10
AGCAACTTCTGTTTTTTTAGCCGTAGGCTGTACATCAACTTCTACTCCTGATTCTGTTGA  300
 A  T  S  V  F  L  A  V  G  C  T  S  T  S  T  P  D  S  V  E   30
AAAGTTTACGAATAACAAAGAAACAGGTGAAGCTCTATTAACGCCAGTAGCCATTACTGC  360
 K  F  T  N  N  K  E  T  G  E  A  L  L  T  P  V  A  I  T  A   50
AAGTAGCCATGATGGTAACGGCCCTGACCGTTTATTCGACCAAGACGTTACAACGCGTTG  420
 S  S  H  D  G  N  G  P  D  R  L  F  D  Q  D  V  T  T  R  W   70
GTCTGCCGCCGGTGATGGCGAATGGGCAATGCTAGACTACGGCTCAGTTCAAGAGTTTGA  480
 S  A  A  G  D  G  E  W  A  M  L  D  Y  G  S  V  Q  E  F  D   90
TGCTGTTCAAGCTGCCTTTAGTAAAGGTAACGAGCGTCAGTCTAAATTTGATATCCAAAT  540
 A  V  Q  A  A  F  S  K  G  N  E  R  Q  S  K  F  D  I  Q  M  110
GAGTGTTGACGGTGAAAGCTGGACCACTGTTTTAGAAGCTCAAGAGAGCTCTGGTAGAGC  600
 S  V  D  G  E  S  W  T  T  V  L  E  A  Q  E  S  S  G  R  A  130
CCTTGGTTTAGAGCGTTTTCAGTTCGAGCCTGCGGTAAATGCCCGCTACGTACGATATGT  660
 L  G  L  E  R  F  Q  F  E  P  A  V  N  A  R  Y  V  R  Y  V  150
AGGACACGGCAACACCAAAAACGGTTGGAACAGTGTAACCGAACTCGCAGCCGTTAACTG  720
 G  H  G  N  T  K  N  G  W  N  S  V  T  E  L  A  A  V  N  C  170
TAGCATTAACGCTTGTCCATCAAGCCACATCATAACTAGTGCAGTAGTTGCCGCTGAAGC  780
 S  I  N  A  C  P  S  S  H  I  I  T  S  A  V  V  A  A  E  A  190
TTCAATGATTGCAGAAATGAAAGCGGCAGAGAAAGCACGTAAAGCAGCGCGTAAAGACCT  840
 S  M  I  A  E  M  K  A  A  E  K  A  R  K  A  A  R  K  D  L  210
TCGTTCAGGTGATTTTGGCGCGCCAGCCGTATACCCTTGTGAAACCACAGTAACGTGTTT  900
 R  S  G  D  F  G  A  P  A  V  Y  P  C  E  T  T  V  T  C  230
GCGTTCTGAGCTTCCTCCAACTCCGGCTTTACCAGAAACGCCACTACCAGGTAACGCGCC  960
 R  S  E  L  P  P  T  P  A  L  P  E  T  P  L  P  G  N  A  P  250
AAGCGAAAACTTTGACCTGTCTTACTGGTACTTATCGCAGCCTTTCGACCACGATAAAAA 1020
 S  E  N  F  D  L  S  Y  W  Y  L  S  Q  P  F  D  H  D  K  N  270
TGAACGCCCAGATGACGTGTCTGAATGGAACCTTGCAAATGGTTATCAGCACCCTGAGAT 1080
 E  R  P  D  D  V  S  E  W  N  L  A  N  G  Y  Q  H  P  E  I  290
CTTCTATACAGCAGACGATGGTGGTTTAGTATTCAAGTCATACGTGAAAGGGGTTCGTAC 1140
 F  Y  T  A  D  D  G  G  L  V  F  K  S  Y  V  K  G  V  R  T  310
CTCTAAAAACACCAAGTATGCGCGTACCGAGCTTCGTGAAATGCTACGTCGCGGTAACAT 1200
 S  K  N  T  K  Y  A  R  T  E  L  R  E  M  L  R  R  G  N  M  330
GTCGCACAAAACTAAAGGTGTGAACAAGAACAACTGGGTATTCTCAAGCGCTCCAGAAGC 1260
 S  H  K  T  K  G  V  N  K  N  N  W  V  F  S  S  A  P  E  A  350
AGACTTAAAAGCTGCCGGCGGTATTGATGGCGTTCTAGAAGCTACCCTTAAAATTGACCA 1320
 D  L  K  A  A  G  G  I  D  G  V  L  E  A  T  L  K  I  D  H  370
CGCCACCACTACTGGTGCGGCTAACGAAGTAGGTCGCTTTATTATTGGTCAAATCCACGA 1380
 A  T  T  T  G  A  A  N  E  V  G  R  F  I  I  G  Q  I  H  D  390
CCAAAACGATGAGCCAATTCGTTTGTACTACCGTAAGCTGCCAAACCAAGCAACTGGTGC 1440
 Q  N  D  E  P  I  R  L  Y  Y  R  K  L  P  N  Q  A  T  G  A  410
AGTTTACTTTGCTCACGAAAGCCAAGACGCTACCAAAGAGGATTTCTATCCGCTAGTAGG 1500
 V  Y  F  A  H  E  S  Q  D  A  T  K  E  D  F  Y  P  L  V  G  430
TGATTTAACGGCTGAGGTTGGTGAAGATGGTATCGCGCTAGGTGAAGTATTTAGCTACCG 1560
 D  L  T  A  E  V  G  E  D  G  I  A  L  G  E  V  F  S  Y  R  450
CATCGAAGTGGTTGGTAACACCATGACGGTTACCGTTTCTCGAGAAGGCAAAGAAGACGC 1620
 I  E  V  V  G  N  T  M  T  V  T  V  S  R  E  G  K  E  D  A  470
TGTTCAGGTAGTTGACATGAGCGACAGTGGCTACGATGTTGGTGGTAAGTACATGTACTT 1680
 V  Q  V  V  D  M  S  D  S  G  Y  D  V  G  G  K  Y  M  Y  F  490
TAAAGCCGGTGTTTACAACCAAAACATCTCTGGCGATTTAGATGATTACTCTCAAGCCAC 1740
 K  A  G  V  Y  N  Q  N  I  S  G  D  L  D  D  Y  S  Q  A  T  510
CTTCTATAAACTAAAAGCTACTCACGATAGTTACACTGAAAAATAAGCTTTAGTTATCGC 1800
 F  Y  K  L  K  A  T  H  D  S  Y  T  E  K  *                524
TTTGTTTAAAAATACCCCGCTGTAAAGCGGGGTATTTTTTTGGCTTGTTAAATTTGCGGC 1860

TTATTTTTGTTGGTAATCTAAACCTAGATAATCGAATACGAAACCCATCAACCACAGCGG 1920
ACCTATTAGTAGGTACTGAATATCTTCAAAGAAGGAAGGTTTAGCGCCTTCTATTTTGTG 1980
TCCGATAAATTGGCCAATCCAAGCGACAACGAAGATTATTAGGCTCACCTGCCATATGGG 2040
CAATTGGGGCTAAATTTGAGTAATCAAATCACAAACCTAAAAAAAGCAAGCTTACAAGTA 2100
AAACCAAGCGCCCATTGGGCTTAAAC                                    2126
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ces and many alginate lyase encoding genes have been cloned from some types of bacteria (Wong et al. 2000). Among them, AlyP of *Pseudomonas* sp. (Maki et al. 1993), AlgL of *Pseudomonas aeruginosa* (Boyd et al. 1993), AlxM of *Photobacterium* sp. (Malissard et al. 1993), AlyVM1 of *Vibrio halioticoli* (Sugimura et al. GenBank AAF22511), Aly of *Pseudomonas alginovora* (Chavagnat et al. 1996), alginate lyase A1-III of *Sphingomonas* sp. A1 (Yoon et al. 2000), and AlyVOA and AlyVOB of *Vibrio* sp. O2 (Kawamoto et al. 2006) are alginate lyases specific for polymannuronate, while AlyA of *Klebsiella pneumoniae* (Baron et al. 1994), AlyPG of *Corynebacterium* sp. ALY-1 (Matsubara et al. 2000), alginate lyase A1-II of *Sphingomonas* sp. A1 (Yoon et al. 2000), AlyVI of *Vibrio* sp. QY101 (Han et al. 2004), Aly of *Streptomyces* sp. ALG-5 (Kim et al. 2009), and AlyVG1, AlyVG2, and AlyVG3 of *V. halioticoli* (Sugimura et al. 2000) are alginate lyases specific for polyguluronate. Although a lyase may be listed as M specific or G specific, some alginate lyases had broad substrate specificity. For example, AlyPEEC of *Pseudoalteromonas elyakovii* IAM 14594 (Sawabe et al. 2001) could degrade both M blocks and G blocks, A1-II′ of *Sphingomonas* sp. A1 degrades all substrates equally (Yamasaki et al. 2005), and A1m of *Agarivorans* sp. favorably degraded the MG and G blocks in alginate (Kobayashi et al. 2009).

In a previous study, we isolated a marine bacterium *Pseudoalteromonas* sp. CY24, which could produce a novel endo-β-agarase AgaB, from seawater of Jiaozhou Bay of Qingdao, China (Ma et al. 2007). Alginate lyase activity was also found in the medium of *Pseudoalteromonas* sp. CY24 when sodium alginate was added to the medium. In this note, we report the cloning, sequence analysis, and expression of gene *alyPI* encoding alginate lyase of *Pseudoalteromonas* sp. CY24 in *Escherichia coli* and purification and characterization of its product.

The alginate lyase encoding gene was cloned from the genomic DNA of *Pseudoalteromonas* sp. CY24 by using a strategy of combined degenerate PCR and inverse PCR. Degenerate primers PalyPI-F1 (5′-CGBTCDGARCTBCGBGMRATG-3′) and PalyPI-R1 (5′-RTARTTRCCBGCYTTRAARTA-3′) were designed corresponding to conserved regions YXRSELREM and YFKAGXYXQ of extracellular alginate lyase genes to amplify a 534 bp DNA fragment using the genomic DNA of *Pseudoalteromonas* sp. CY24 as template. The PCR product was purified and sequenced. FASTA searches revealed that the 534 bp fragment had homology to published alginate lyase genes. To obtain the remainder of the *alyPI* gene, an inverse PCR technique was carried out using the specific primers PalyPI-F2 (5′-TCGTTTTGGTCGTGGATTTG-3′) and PalyPI-R2 (5′-GGCTGAGGTTGGTGAAGATG-3′) designed from the obtained degenerate PCR fragment. Inverse PCR amplification generated a 4.2 kb fragment and sequence analysis showed an overlapping region with the previously sequenced 534 bp PCR fragment.

As shown in Fig. 1, translation of the possible reading frames revealed only one complete open reading frame of 1575 bp that started with an ATG codon at position 212 and terminated with the TAA codon at position 1786. A putative AGGTGAA ribosome-binding site occurred 7 bp upstream from the ATG start codon. The sequences of TTGAAA and GATAAT located 89 bp upstream of the ribosome-binding site could correspond to the –35 and –10 boxes of a promoter. An inverted repeat AAAAATACCCCGCT and AGCGGGGTATTTTT with a GTAA loop located 21 bp downstream of the TAA stop codon

Fig. 2. Alignment of the predicted AlyPI protein with the putative alginate lyase from *Vibrio splendidus* 12B01 (GenBank accession No. ZP 00990010). Asterisks denote identical residues in two sequences and colons and dots denote conservative substitutions. The two conserved nine amino acid regions are underlined.

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AlyPI        MKHIYLKSLLATSVFLAVGCTSTSTPDSVEKFTNNKETGEALLTPVAITASSHDGNGPDR 60
ZP_00990010  MKQITLKTLLASSILLAVGCASTSTP--TADFPNNKETGEALLTPVAVSASSHDGNGPDR 58
             **:* **:***:*::*****:*****  . .*.***************::***********

AlyPI        LFDQDVTTRWSAAGDGEWAMLDYGSVQEFDAVQAAFSKGNERQSKFDIQMSVDGESWTTV 120
ZP_00990010  LVDQDLTTRWSSAGDGEWATLDYGSVQEFDAVQASFSKGNQRQSKFDIQVSVDGESWTTV 118
             *.***:*****:******* ***************:*****:********:**********

AlyPI        LEAQESSGRALGLERFQFEPAVNARYVRYVGHGNTKNGWNSVTELAAVNCSINACPSSHI 180
ZP_00990010  LENQLSSGKAIGLERFQFEPVVQARYVRYVGHGNTKNGWNSVTGLAAVNCSINACPASHI 178
             ** * ***:*:*********.*:******************** ************:***

AlyPI        ITSAVVAAEASMIAEMKAAEKARKAARKDLRSGDFGAPAVYPCETTVTC-LRSELPPTPA 239
ZP_00990010  ITSDVVAAEAVIIAEMKAAEKARKDARKDLRSGNFGVAAVYPCETTVECDTRSALPVPTG 238
             *** ****** :************ ********:**..********* *  ** ** ...

AlyPI        LPETPLPGNAPSENFDLSYWYLSQPFDHDKNERPDDVSEWNLANGYQHPEIFYTADDGGL 299
ZP_00990010  LPATPVAGNSPSENFDMTHWYLSQPFDHDKNGKPDDVSEWNLANGYQHPEIFYTADDGGL 298
             ** **:.**:******:::************ :**************************

AlyPI        VFKSYVKGVRTSKNTKYARTELREMLRRGNMSHKTKGVNKNNWVFSSAPEADLKAAGGID 359
ZP_00990010  VFKAYVKGVRTSKNTKYARTELREMMRRGDQSISTKGVNKNNWVFSSAPESDLESAAGID 358
             ***:**********************:***: * .*****************:**::*.***

AlyPI        GVLEATLKIDHATTTGAANEVGRFIIGQIHDQNDEPIRLYYRKLPNQATGAVYFAHESQD 419
ZP_00990010  GVLEATLKIDHATTTGNANEVGRFIIGQIHDQNDEPIRLYYRKLPNQETGAVYFAHESQD 418
             *************** ****************************** ************

AlyPI        ATKEDFYPLVGDLTAEVGEDGIALGEVFSYRIEVVGNTMTVTVSREGKEDAVQVVDMSDS 479
ZP_00990010  ATKEDFYPLVGDMTAEVGDDGIALGEVFSYRIDVKGNTMTVTLIREGKDDVVQVVDMSNS 478
             ************:*****:*************:* *******: ****:*.*******:*

AlyPI        GYDVGGKYMYFKAGVYNQNISGDLDDYSQATFYKLKATHDSYTEK 524
ZP_00990010  GYDAGGKYMYFKAGVYNQNISGDLDDYSQATFYQLDVSHDQYKK- 522
             ***.*****************************:*..:**.*.:
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Fig. 3. SDS–PAGE analysis of purified AlyPI from culture supernatant of *Escherichia coli* BL21-pET24-alyPI. The standards of molecular mass and the purified enzyme are shown to the left and right of the gel plate, respectively. The protein was stained with Coomassie brilliant blue R-250.

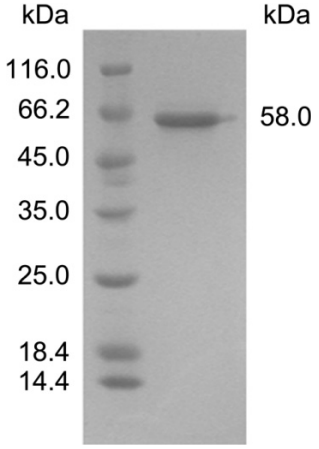
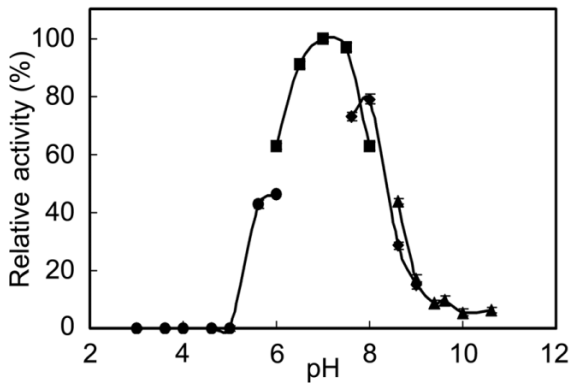


Fig. 4. Optimal pH of AlyPI. A 0.9 mL amount of alginate solution (3 mg/mL) with different pHs was incubated at 40 °C for 10 min before addition of 0.1 mL of the enzyme. The activity at various pHs was assayed as described in the Materials and methods. Buffers used: circles, 50 mmol/L citrate buffer; squares, 50 mmol/L phosphate buffer; diamonds, 50 mmol/L Tris–HCl; triangles, 50 mmol/L glycine–NaOH.



might function as a terminator region. The deduced product AlyPI of 524 amino acid residues has a theoretical molecular mass of 57.4 kDa and a pI value of 4.99 using the Compute pI/Mw tool (http://us.expasy.org/tools/pi_tool.html). The region M^1 to T^{23} was estimated as a putative signal peptide by using the SignalP program (http://www.cbs.dtu.dk/

Fig. 5. pH stability of AlyPI. After incubation of the enzyme at 4 °C for 24 h with different pHs, the remaining activity was assayed as described in the Materials and methods. Buffers used: circles, 50 mmol/L citrate buffer; squares, 50 mmol/L phosphate buffer; diamonds, 50 mmol/L Tris–HCl; triangles, 50 mmol/L glycine–NaOH.

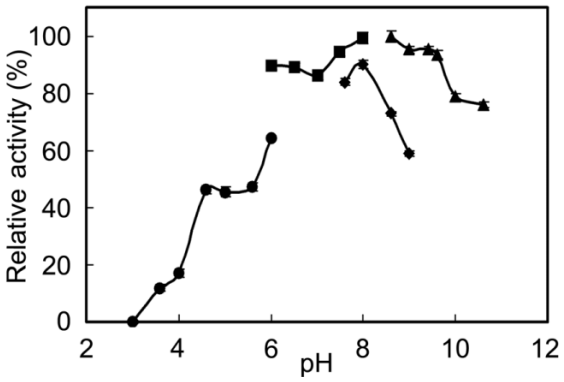


Table 1. Effects of chemical reagents on AlyPI activity.

Reagent	Concentration (mmol/L)[a]	Relative activity (%)[b]
None	—	100
NaCl	50	181
	100	280
	200	244
	300	208
	500	109
	700	76
	900	59
KCl	1	96
	10	92
	50	117
	100	67
$(NH_4)_2SO_4$	1	96
EDTA	1	56
SDS	1	35
$NiCl_2$	1	67
$FeCl_3$	1	147
$BaCl_2$	1	79
$MnCl_2$	1	150
$CaCl_2$	1	136
$MgCl_2$	1	87
$ZnCl_2$	1	68

[a]The final concentration of each reagent in the assay mixture.

[b]Values are the mean of three independent determinations and expressed as a percentage of control activity (set as 100%).

services/SignalP/). It was a new member of alginate lyases from a marine bacterium and was classed into the ~60 kDa group according to their molecular mass. The alginate lyase gene has been submitted to the GenBank nucleotide sequence database under accession No. FJ643537.

The deduced amino acid sequence of AlyPI was compared with other known alginate lyases by a homology search in GenBank using the BLAST program. The analysis revealed little homology between AlyPI and the other alginate lyases except a putative alginate lyase from *Vibrio splendidus* 12B01 (GenBank accession No. ZP 00990010), which showed 84% identity and 91% similarity to AlyPI (Fig. 2). However, the characterization of this putative alginate lyase remains unknown. The region YFKAGXYXQ, regarded as a striking feature at the C termini of several alginate lyase of ~30 kDa (Wong et al. 2000), was observed in the region Y^{489}–Q^{497} of AlyPI, which was classed into the ~60 kDa group of alginate lyases. This region was found in several alginate lyases with different substrate specificities, which indicated the irrelevancy of this consensus region to M or G O-linkage recognition and importance in maintaining the stable three-dimensional conformation of the lyases, as suggested by Malissard et al. (1993).

To express the AlyPI protein, the primers were designed to introduce the *Nde*I (PalyPI-F3: GGAATTCCATATGAAACATATCTATCTTAA) and *Eco*RI (PalyPI-R3: GGAATTCGATTTTTCAGTGTAACTATCGT) restriction sites encompassing *alyPI*. An expected PCR product of 1.6 kb was amplified, purified, digested with *Nde*I and *Eco*RI, and ligated into a similarly digested pET-24a (+). The resulting plasmid, pET24-alyPI, was transformed to *E. coli* BL21 (DE3) to express the *alyPI* gene with its own signal peptide encoding sequence and the His-tag encoding sequence fused at the 3′ end. *Escherichia coli* BL21 (DE3) cells harboring plasmid pET24-alyPI were cultured in LB medium supplemented with 30 μg/mL kanamycin at 37 °C to an absorbance at 600 nm (OD_{600}) of 0.5 and then isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mmol/L. After incubation at 25 °C for an addtional 18 h, the culture supernatant was harvested by centrifugation (10 000*g* for 10 min), dialysed against buffer A (20 mmol/L phosphate buffer (pH7.0) and 500 mmol/L NaCl), filtered through a 0.22 μm pore size filter, and loaded on a HisTrap HP column (Amersham Biosciences, USA) preequilibrated with buffer A. Inactive proteins were eliminated by washing the column with buffer A containing 20 mmol/L imidazole and alginate lyase protein was eluted with buffer A containing 100 mmol/L imidazole. The active fractions were dialysed against 50 mmol/L phosphate buffer (pH 7.0) and stored at –20 °C.

Alginate lyase activity was measured by an increase in absorbance at 235 nm of the reaction products using a 1 cm cuvette. Unless stated otherwise, 0.1 mL of the lyase was added to 0.9 mL of substrate solution (3 mg/mL in 50 mmol/L phosphate buffer, pH7.0) and incubated at 40 °C for 10 min. One unit of the enzyme activity was defined as an increase of 1.0 in absorbance at 235 nm/min. The specific activity of 121.6 units/mg in 50 mmol/L phosphate buffer (pH 7.0) and a final yield of 81% were achieved. The purified AlyPI gave a single band on SDS–PAGE with a molecular mass of 58.0 kDa (Fig. 3), which is in good agreement with the value estimated from the deduced amino acid sequence of AlyPI S^{24} to K^{524} with the fusion motif.

The optimal temperature for AlyPI was 40 °C. The thermal stability of AlyPI was investigated by measuring the residual activity at 40 °C after the enzymes were incubated at various temperatures in 50 mmol/L phosphate buffer (pH 7.0) for 1 h. The residual activity decreased when the temperature went above 20 °C and disappeared at 50 °C. The

effects of pH on activity and stability of AlyPI were measured using 50 mmol/L citrate buffer (pH 3–6), 50 mmol/L phosphate buffer (pH6–8), 50 mmol/L Tris–HCl buffer (7.6–9), and 50 mmol/L glycine and NaOH (pH 8.6–10.6). The enzyme was most active at pH 7.0, as shown in Fig. 4, and was stable over a pH range of 6.0–10.6 with more than 50% of maxium activity maintained when incubated in buffers with various pHs at 4 °C for 24 h (Fig. 5). The activity of the enzyme was obviously enhanced in the presence of Na^+, Mn^{2+}, Ca^{2+}, and Fe^{3+} but significantly inhibited by SDS and EDTA. NaCl enhanced the activity of the enzyme by 2.8 times at 100 mmol/L but inhibited the activity when the concentration was greater than 700 mmol/L (Table 1).

The substrate specificity of the alginate lyase AlyPI was measured using equal molar amounts of poly-M blocks, poly-G blocks, and sodium alginate (measured as sugar residues). The results showed that AlyPI could degrade all three types of substrates and showed higher degradation activity for sodium alginate. Poly-M blocks gave 78% and poly-G blocks gave 46% of the activity of sodium alginate. Therefore, the alginate lyase AlyPI was shown to be a broad substrate lyase. The kinetics data were obtained by continuously monitoring the absorbance at 235 nm of the reactions at 40 °C in a 1 cm cuvette using 10 different concentrations of substrate ranging from 0.7 to 7 mmol/L (sugar residues). The K_m value of AlyPI for sodium alginate was found to be 2.36 mmol/L (sugar residues) using HYPER.EXE analysis.

As the typical characteristic of the G-specific lyases, the consensus nine amino acid region YXRESLREM was previously only found in the N-terminus region of G-specific lyases, but this view was revised and complemented by the reports on alginate lyases containing this consensus region of broad substrate specificity, such as AlyVI of *Vibrio* sp. QY101, the first reported alginate lyase of broad substrate specificity harboring this consensus region (Han et al. 2004), A1-II′ of *Sphingomonas* sp. A1 (Yamasaki et al. 2005), and an MG- and G-specific alginate lyase A1m of *Agarivorans* sp. (Kobayashi et al. 2009). As a broad substrate lyase, an amino acid region, YARTELREM, instead of the consensus region YXRSELREM was also observed in the N-terminus region of AlyPI corresponding to the region Y^{316}–M^{324}. Thus far, we have found that all reported alginate lyases containing this consensus region could degrade G blocks, and alginate lyases that could only degrade M blocks did not contain this region. Although the region YXRSELREM existed in some alginate lyases with broad substrate specificity, it was more consensus in G-specific lyases, and the consensus sequence RXELR other than YXRSELREM existed in the N-terminus of three M-specific lyases (AlxM, AlyVOA, and AlyVOB). Further research should be done to elucidate the relationship between the substrate specificity and the two consensus regions (YXRSELREM and RXELR).

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