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Comparative characterization of three bacterial exo-type alginate lyases

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ABSTRACT

Alginate, a major acidic polysaccharide in brown macroalgae, has attracted attention as a carbon source for production of ethanol and other chemical compounds. Alginate is monomerized by exo-type alginate lyase into an unsaturated uronate; thus, this enzyme is critical for the saccharification and utilization of alginate. Although several exo-type alginate lyases have been characterized independently, their activities were not assayed under the same conditions or using the same unit definition, making it difficult to compare enzymatic properties or to select the most suitable enzyme for saccharification of alginate. In this study, we characterized the three bacterial exo-type alginate lyases under the same conditions: A1–IV of *Sphingomonas* sp. strain A1, Atu3025 of *Agrobacterium tumefaciens*, and Alg17c of *Saccharophagus degradans*. A1–IV had the highest specific activity as well as the highest productivity of uronate, whereas Alg17c had the lowest activity and productivity. Only dialyzed Atu3025 and Alg17c were tolerant to freezing. Alg17c exhibited a remarkable halotolerance, which may be advantageous for monomerization of alginate from marine brown algae. Thus, each enzyme exhibited particular desirable and undesirable properties. Our results should facilitate further utilization of the promising polysaccharide alginate.

1. Introduction

Alginate is a major acidic polysaccharide in brown macroalgae. For example, fronds of the brown algae *Laminaria digita* contain 25–44% alginate (by dry weight); stipes of *L. digita,* 35–47%; fronds of *Laminaria hyperborea,* 17–33%; stipes of *L. hyperborea,* 25–38%; and genus *Sargassum*, 3.3–41% [1,2]. Alginate consists of β-D-mannuronate (M) and its C5 epimer α-L-guluronate (G), arranged as polyM, polyG, and heteropolymeric random sequences (polyMG) [3]. Recently, alginate has attracted attention as a carbon source for production of ethanol and other chemical compounds, largely because of the advantages of brown macroalgae over land crops: higher productivity; no requirement for arable land, irrigation water, or fertilizer; and the absence of lignin [4–7]. Takeda et al. initially succeeded in producing ethanol from alginate using a bioengineered bacterium, *Sphingomonas* sp. strain A1 [8]. Subsequently, ethanol production from alginate and mannitol, another major carbohydrate in brown macroalgae, was achieved using bioengineered *Escherichia coli* [9,10]. We also established a system for producing pyruvate from alginate using *Sphingomonas* sp. strain A1 [11].

In *Sphingomonas* sp. strain A1, alginate is depolymerized by endo-type alginate lyases (A1–I, A1–II, and A1–III) to produce oligoalginates [12], which are then degraded by exo-type alginate lyase (A1–IV) into an unsaturated uronate that is further non-enzymatically converted to 4-deoxy-L-erythro-5-hexoseulose uronate (DEH) [13,14]. DEH is reduced to 2-keto-3-deoxy-D-gluconate (KDG) and metabolized to pyruvate [15,16] (Fig. 1). Alginate is also directly monomerized to DEH by the exo-type alginate lyase. Thus, exo-type alginate lyase is a key enzyme for the saccharification and utilization of alginate. Moreover, Enquist-Newman et al. recently created a bioengineered yeast *Saccharomyces cerevisiae* that can produce ethanol from both mannitol and DEH [17], highlighting the significance of the exo-type alginate lyase that produces DEH; industrial use of the exo-type alginate lyase enables us to prepare an industrial scale of DEH from alginate.

Several bacterial exo-type alginate lyases have been well characterized, including A1–IV of *Sphingomonas* sp. strain A1 (an alginate-assimilating bacterium) [13,14], Atu3025 of *Agrobacterium tumefaciens* (the causal bacterium of crown gall) [18,19], Alg17c of *Saccharophagus degradans* (a marine carbohydrate-degrading bacterium) [20–23], OalA, OalB, and OalC of *Vibrio*

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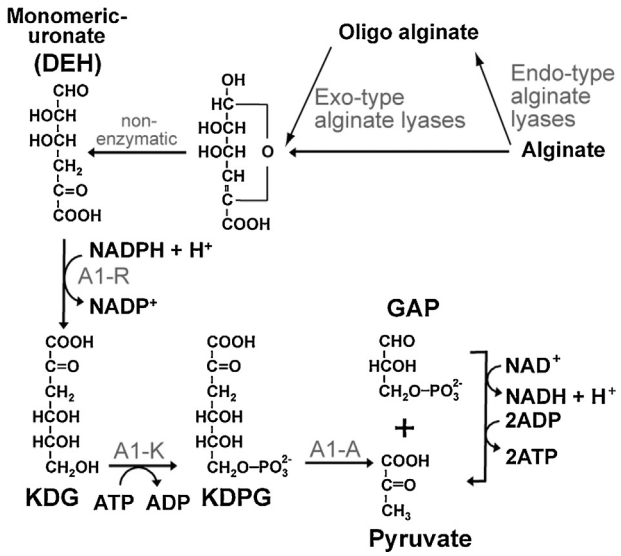

Fig. 1. Alginate metabolism. Details are described in the text.

splendidus (a marine bacterium) [24], and AlyA5 of *Zobellia galactanivorans* (a marine bacterium) [25]. A1–IV, Atu3025, and OalA are classified as PL–15, AlyA5 is PL–7, whereas Alg17c, OalB, and OalC are PL–17. A1–IV was the first of these enzymes to be identified [13]. The tertiary structures of Atu3025 and Alg17c have been solved [21,26], enabling the functions of these enzymes to be improved based on their structural information. Recently, Alg7K of *S. degradans*, belonging to PL7 [27], has been successfully expressed on the cell surface of *S. cerevisiae* and demonstrated to show the exo-type alginate lyase activity [28].

Although several exo-type alginate lyases have been characterized independently, their activities have not been assayed under the same conditions or using the same unit definition, making it difficult to compare their enzymatic properties or to select the most suitable enzyme for saccharification of alginate (Table 1). Therefore, we decided to characterize three bacterial exo-type alginate lyases, A1–IV, Atu3025, and Alg17c, under the same conditions in order to compare their activities. These 3 lyases were selected, since A1–IV is the first exo-type alginate lyase identified [13] and the tertiary structures of Atu3025 and Alg17c have been solved [21,26].

2. Materials and methods

2.1. Materials

Sodium alginate (viscosity 1,000 cps, from *Eisenia bicyclis*) was purchased from Nacalai Tesque (Kyoto, Japan). DEH was prepared as described [16].

2.2. Plasmid construction

Primers and plasmids used in this study are listed in Tables 2 and 3, respectively. The genes encoding Alg17c, Atu3025, and A1–IV were amplified by PCR using KOD-Plus Neo (Toyobo, Osaka, Japan) in the presence of 8% v/v DMSO and 3.0 mM $MgSO_4$. Templates, primers, and annealing temperatures for amplification of the genes for Atu3025, Alg17c, and A1–IV were as follows. Atu3025: pMK4544, primers 1 and 2, 54 °C; Alg17c: pMK5306, primers 3 and 4, 52 °C; A1–IV: genomic DNA of *Sphingomonas* sp. strain A1 [29], primers 5 and 6, 53 °C. The three amplified genes were inserted into pET-21b digested with *Nde*I and *Not*I using In-Fusion (Takara, Otsu, Japan), yielding pMK5400 (Atu3025), pMK5399 (Alg17c), and pMK5398 (A1–IV).

2.3. Expression and purification of Alg17c, Atu3025, and A1-IV

pMK5400 and pMK5398 were introduced into *E. coli* HMS174(DE3), yielding strains MK5407 and MK5405. pMK5399 was into *E. coli* BL21(DE3), yielding strain MK5406. Alg17c, Atu3025, and A1–IV were expressed in MK5406, MK5407, and MK5405 as described [14,18,20] with slight modifications: expression was induced by addition of 0.1 mM isopropyl β-D-1-thiogalactopyranoside, and the cells were cultivated for 20–24 h at 16 °C (Alg17c in MK5406) or at 30 °C (Atu3025 in MK5407 and A1–IV in MK5405). Alg17c, Atu3025, and A1–IV were expressed as C-terminally His-tagged fusion proteins. The expressed Alg17c lacks a 22 amino-acid N-terminal sequence (^{2}LSVNTIKNTLLAAVLVSVPATA^{23}) [20].

Cells were disrupted by sonication, and expressed proteins were purified on a TALON Metal Affinity Resin column (Clontech, Otsu, Japan). The column was washed with 20 mM Tris–HCl (pH 7.5) plus 300 mM NaCl, followed by elution with 20 mM Tris–HCl (pH 7.5) plus 300 mM NaCl and 150 mM neutralized imidazole, as described [30].

Table 1
Comparisons of assay conditions and properties of three exo-type alginate lyases from bacteria.

	Alg17c [19]	Atu3025 [17]	A1–IV [16]
Reaction mixture for assay	20 mg/ml sodium alginate, 20 mM Tris–HCl (pH 6.0), total 100 μl	0.5 mg/ml sodium alginate, 50 mM Tris–HCl (pH 7.5), total 1.0 ml	0.42 mg/ml alginate trisaccharide, 50 mM Tris–HCl (pH 7.5), total 10 μl
Amount of purified protein in the reaction mixture	50.6 nmol (4082 μg) in 100 μl	NR[a]	2.0 μg in 10 μl
Reaction temperature and time	40 °C, 30 min	30 °C, 5 min	30 °C, 10 min
Definition of one unit	The amount of enzyme required to release 1.0 μmol of the reducing sugar (based on glucose)	The amount of enzyme required to release 1.0 μmol of β-formylpyruvic acid per min	The amount of enzyme required to degrade 1.0 μmol of trisaccharide per min
Analytical method	DNS method	TBA method	TLC analysis
Protein assay	NR	Based on molecular coefficient at A_{280}	Based on A_{280} assuming A_{280} = 1 corresponds to 1.0 mg/ml
Source of sodium alginate	Sigma–Aldrich	Nacalai Tesque	Nacalai Tesque
Tertiary structure	Available	Available	Unavailable
K_m (mM)	35.2 mg/ml	0.24 mg/ml	NR
V_{max} (U/mg)	41.7 U/mg	20.5 U/mg	NR
Optimum temperature	40 °C	30 °C	37 °C
Optimum pH	pH 6.0 (50% at pH 7.5)[b]	pH 7.3	pH 7.5–8.5

[a] Not reported in the reference.
[b] Alg17c had 50% activity at pH 7.5 and 100% activity at pH 6.0.