Biochemistry (Moscow), Vol. 69, No. 3, 2004, pp. 262-269. Translated from Biokhimiya, Vol. 69, No. 3, 2004, pp. 328-337.

Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM03-001, October 15, 2003.

Intracellular Alginolytic Enzymes of the Marine Bacterium *Pseudoalteromonas citrea* KMM 3297

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Received December 24, 2002
Revision received April 15, 2003

Abstract—The marine bacterium *Pseudoalteromonas citrea* KMM 3297 is an associate of the holothurian *Apostichopus japonicus*. When grown in a medium containing glucose, the strain produces two intracellular alginolytic enzymes, AlI and AlII. Fucoidan from the brown alga *Fucus evanescens* induces synthesis of one more alginolytic enzyme, AlIII. These enzymes were separated using anion-exchange chromatography. The alginate lyase AlI completely retains its activity at 35°C, AlII and AlIII being stable at 45°C. The alginate lyases exhibit maximal activities in the range of pH 7-8. The molecular weights of AlI, AlII, and AlIII determined by gel filtration are 25, 79, and 61 kD, respectively. All the investigated enzymes are endo-type alginate lyases. They catalyze degradation of polyguluronate (poly-G) and polymannuronate (poly-M) yielding oligosaccharides of the polymerization degree of $5 \geq n \geq 3$ with the unsaturated bond between the C4 and C5 atoms of the non-reducing terminus. A mixture of these three enzymes exhibits synergism while acting on the polymeric substrate. The K_m values of the alginate lyase AlI for poly-G and poly-M are 24 and 34 μg/ml, respectively. Alginate lyase AlIII exhibits less affinity to poly-M (K_m = 130.0 μg/ml) than to poly-G (K_m = 40.0 μg/ml). NaCl (0.2 M), $MgCl_2$ and $MgSO_4$ (0.01 M) activate all three enzymes more than twofold. The presence of several alginolytic enzymes of different specificity provides efficient destruction of alginic acids of brown algae by the strain *P. citrea* KMM 3297.

Key words: alginate lyase, marine bacterium, *Pseudoalteromonas citrea*, alginic acid, brown algae

Alginic acids are linear polysaccharides composed of the residues of β-D-mannuronic and α-L-guluronic acids that are bound with 1,4-O-glycoside bonds [1].

They are widely used in different fields of industry, agriculture, and medicine [2, 3]. The main sources of alginic acid are the brown algae *Laminariaceae* and *Fucaceae* [2].

Alginate lyases—mannuronate lyases (EC 4.2.2.3) and guluronate lyases (EC 4.2.2.11)—catalyze degradation of alginic acids by the β-elimination mechanism yielding a double bond between the C4-C5 atoms of the monosaccharide residue on the non-reducing terminal of an oligouronide. These enzymes have been isolated from marine algae, mollusks, microorganisms, and soil bacteria. Japanese researchers have isolated and characterized several species of alginolytic bacteria from brown algae grown in mariculture [3, 4]. These bacteria are usually pathogenic for the algae, and the extent of their pathogenicity is determined by their ability to synthesize highly active extra- and intracellular alginate lyases [3].

Alginate lyases are used for investigation of the structure of alginates and alginate-containing polysaccharides [5]. They are also successfully used to obtain protoplasts of different algae [6, 7]. The use of highly selective alginate lyases together with natural alginates produce polymannuronic (poly-M), polyguluronic (poly-G), and also polymannuro-guluronic (poly-MG) blocks that are employed for investigations of the substrate specificity of the enzymes. There is also a possibility of using the alginate lyases together with deoxyribonuclease and antibiotics as therapeutic preparations to treat diseases caused by pathogens synthesizing mucoid layers with a high content of alginate [5].

Alginate lyases from a number of marine *Pseudoalteromonas*-like bacteria (epibionts of brown algae [3, 4]) and bacterial associates of mollusks [8] have been best studied. Most of the enzymes capable of hydrolyzing alginic acids are lyases, although the marine fungus *Asteromyces cruciatus* together with alginate lyase produces alginate hydrolase [9].

Previously we showed that the marine bacterium *Pseudoalteromonas citrea* KMM 3297 [10] isolated from

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the coelomic liquid of the holothurian *Apostichopus japonicus* synthesized enzymes capable of cleaving polyanionic polysaccharides of brown algae—fucoidan and alginic acid [11]. The present work is devoted to investigation of the alginolytic enzymes of *P. citrea* KMM 3297, an associate of holothuria.

MATERIALS AND METHODS

Materials. Peptone and yeast extract were from Difco (USA); casein hydrolyzate, glucose, xylose, and glucuronic acid were from Merck (Germany); bovine serum albumin, ovalbumin, anhydrase, chymotrypsinogen A, myoglobin, cytochrome *c*, aprotinin, dextrans T-10, T-20, T-40, T-80, DEAE-Sepharose CL-6B, and Sepharose CL-6B were from Sigma (USA); FPLC columns Superdex 75-HR, Source 15Q PE, and Sephadex G-10 were from Pharmacia (Sweden); polyguluronic acid was from The British Drug Houses, Ltd. (England). Polymannuronic acid and fucoidan were isolated from the brown alga *Fucus evanescens* as described in [12].

Analytical methods. The concentration of reducing sugars was determined by the Nelson method [13]. Total sugar was determined using the phenol–sulfuric acid method [14]. Protein concentration was measured by the Lowry method [15].

Microorganisms and conditions of their cultivation. We used a strain of the marine aerobic proteobacterium *Pseudoalteromonas citrea* KMM 3297 isolated from the coelomic liquid of the holothurian *Apostichopus japonicus*.

The *P. citrea* KMM 3297 strain was cultivated in different media of the following composition. Medium A (g/liter): peptone, 5.0; yeast extract, 2.0; casein hydrolyzate, 2.0; $MgSO_4$, 0.05; glucose, 1.0; sea water, 500 ml; distilled water, 500 ml, pH 7.8. In media B and C, glucose was replaced with xylose or fucoidan, 2.0 or 1.0 g/liter, respectively.

To study the dynamics of bacterial growth and biosynthesis of alginate lyases, the inoculum of *P. citrea* KMM 3297 was grown in 250-ml shaken flasks containing 100 ml of nutrient media A, B, or C for 24 h at 25°C at 150 rpm. The cells were grown until the density of the cell suspension reached 10^9 cells/ml. The resulting seeding material (10^9 cells/ml) was inoculated into twenty 250-ml flasks containing 100 ml of the same nutrient medium. The culture was grown for 60 h in a thermostatted shaker at 25°C and 120 rpm. Samples were taken from one of the flasks every 3 h. Growth of bacteria was monitored spectrophotometrically by measuring the absorption at 560 nm. The cell suspension was centrifuged at 5000*g* for 30 min. The supernatant was assayed for reducing sugars, total sugar, and total protein. The pellet containing fresh bacterial biomass was suspended in 0.01 M phosphate buffer, pH 7.2, and the bacterial cells were broken using a UZDN-2 ultrasonic disintegrator (22 kHz). To extract proteins, the suspension was incubated at 4°C for 3 h, and then centrifuged at 10,000*g* for 30 min. The extract was dialyzed against 0.01 M phosphate buffer, pH 7.2, and assayed for alginolytic activity.

The composition of the alginolytic enzymes in the extracts of *P. citrea* KMM 3297 grown in the presence of fucoidan and glucose was analyzed using high-performance chromatography (AKTA, Germany) on a Source 15Q PE anion-exchange column (100 × 4.6 mm).

Assaying of alginase activity. To assay the alginase activity, the following standard procedure was used. The reaction mixture containing 0.2 ml of alginic acid (aqueous solution, 1 mg/ml), 0.2 ml of 0.1 M phosphate buffer, pH 7.2, and 0.1 ml of the assayed enzyme solution was incubated for 1 h at 20°C, and then the reaction was stopped by the addition of the Nelson reagents [13]. The absorption of the colored solutions was measured at 750 nm (A_{750}). Concentration of the reducing sugars was calculated from a calibration plot using glucuronic acid as the standard.

The unit of activity (U) was defined as the amount of the enzyme catalyzing the formation of 1 μmol of reducing sugars (calculated per glucuronic acid) per 1 h under the conditions indicated. Specific activity was calculated as the enzyme activity (U) per 1 mg protein.

The alginolytic activity was determined using polyguluronic acid as the main substrate. The substrate specificity of the alginate lyases was investigated using polyguluronic and polymannuronic acids.

Isolation of the alginolytic enzymes. Fresh biomass of the bacteria (40 g) grown in medium C (26 h) was suspended in 200 ml of 0.1 M phosphate buffer, pH 7.2. The bacterial cells were broken using a UZDN-2 ultrasonic disintegrator (22 kHz). The suspension was incubated at 4°C for 3 h and then centrifuged at 10,000*g* for 30 min. The extract was fractionated with ammonium sulfate as follows: fraction I, 1-30%; fraction II, 30-70%; fraction III, 70-90% saturation. The pellet was separated by centrifugation, dissolved in 0.01 M phosphate buffer, pH 7.2, dialyzed against the same buffer for 18 h, and the resulting solution was assayed for alginase activity. Fraction II that contained the alginase activity was applied to a DEAE-Sepharose CL-6B column (12.5 × 3 cm) equilibrated with the same buffer, and then proteins were eluted with a linear gradient of NaCl (500 ml of 0.01 M phosphate buffer and 500 ml of 0.5 M NaCl) at 0.5 ml/min (fraction volume, 5 ml). Fractions containing the alginase activity designated as AlI, AlII, and AlIII were concentrated by ultrafiltration using a PM-10 membrane (Amicon, Holland). Fractions AlI, AlII, and AlIII were gel filtered separately on a Sepharose CL-6B column (75 × 3 cm) equilibrated with 0.01 M phosphate buffer, pH 7.2 (0.8 ml/min, fraction volume, 5 ml).

The enzymes separated according to this procedure were used in the subsequent work. The enzymes were

stored at 4°C in 0.01 M phosphate buffer, pH 7.2, containing 0.05% sodium azide.

Molecular weights of the enzyme were estimated by gel filtration using an FPLC analyzer (AKTA) on a Superdex 75-HR column (30 × 1.0 cm). Proteins were eluted with 0.01 M phosphate buffer, pH 7.2, containing 0.15 M NaCl at 0.4 ml/min. Fractions of 0.2 ml were collected. Bovine serum albumin (66 kD), ovalbumin (43 kD), anhydrase (29 kD), chymotrypsinogen A (25 kD), myoglobin (17.6 kD), cytochrome *c* (12.4 kD), and aprotinin (6.5 kD) were used as the markers. Proteins were detected by measuring the absorption at 280 nm using a UV detector (LKB Biochrom, Sweden). The alginolytic enzymes were detected by their enzymatic activity.

Determination of pH optima. The reaction mixture contained 0.2 ml of the substrate (1 mg/ml), 0.2 ml of 0.2 M phosphate buffer, pH 5.5-8.0, and 0.1 ml of the enzyme (after the last step of purification). The enzymatic activity was determined at 20°C after 1 h of incubation.

Thermal stability. The enzyme solution (0.4 ml in 0.1 M phosphate buffer, pH 7.2) was incubated for 20 min at temperatures in the range 10-75°C with an interval of 5°C. The samples were cooled to 20°C, and the enzymatic activity was determined using the standard procedure.

Effect of NaCl and bivalent metal ions. Two hundred microliters of 0.01 M phosphate buffer, pH 7.2, containing 0.2 M NaCl or 0.01 M EDTA or 0.01 M metal salt was supplemented with 0.2 ml of the substrate (2 mg/ml) and 0.1 ml of the enzyme. The alginase activity was determined after 2 h of incubation.

Molecular weight of alginic acids was estimated by gel filtration on a 75-HR Superdex column (30 × 1.0 cm) equilibrated with 0.01 M phosphate buffer, pH 7.2, containing 0.15 M NaCl. A solution of alginic acid (0.1 ml, concentration 10 mg/ml) in the indicated buffer was applied to the column and eluted with the same buffer at 0.4 ml/min (fraction volume, 0.2 ml). Sugars were detected using the phenol–sulfuric acid method. Dextrans with the molecular weights of 10, 20, 40, and 80 kD were used as the standards.

Conjoint action of the alginolytic enzymes on polyguluronic acid. A 1-cm spectrophotometric cuvette containing 0.85 ml of the substrate solution (1 mg/ml) in 0.1 M phosphate buffer, pH 7.2, was supplemented with either 0.05 ml of one of the enzymes and 0.1 ml of 0.01 M phosphate buffer, or 0.15 ml of the enzyme mixture composed of the equal volumes (0.05 ml each). The enzymatic activity was monitored by an increase in ΔA_{235} using the LKB Biochrom spectrophotometer.

Determination of K_m value. A 1-cm spectrophotometric cuvette containing 0.8 ml of a solution of the substrate (0.0125-1.0 mg/ml) in 0.1 M phosphate buffer, pH 7.2, was supplemented with 0.2 ml of the enzyme solution. Formation of the double bond was monitored by measuring the optical density at 235 nm (A_{235}) during 1.5 h at 20°C with time intervals of 5-10 min. The Michaelis–Menten constant (K_m) was calculated using the Lineweaver–Burk method.

Products of the enzymatic reaction. Samples containing the individual enzymes AlI (5 U), AlII (3 U), or AlIII (5 U) in 15 ml of 0.1 M phosphate buffer, pH 7.2, or 15 ml of the enzyme mixture composed of equal volumes (5 ml) of the indicated enzymes were added to 30 mg of polyguluronic or polymannuronic acid. The mixtures were incubated for 24 h, and then the reaction was stopped by boiling for 10 min. The mixtures were centrifuged, desalted on a Sephadex G-10 column (50 × 1 cm), and lyophilized. The products were analyzed using a Jeol JLC-6AH automatic liquid chromatograph (Japan) on a Biogel P-2 column (100 × 1 cm). The products were eluted with 0.05 M acetate buffer, pH 5.2-5.5, containing 0.2 M NaCl at 7-9 ml/h. Sugars were determined using the orcinol–sulfuric acid method [16].

UV absorption spectra of the digestion products of the alginic acids were recorded on a Cecil 7000 CE 7250 spectrophotometer (Cecil, Great Britain).

^{13}C-NMR spectra of alginic acids and the products of their hydrolysis were recorded on a Brucker WM-250 instrument (Bruker, Germany) at 60°C using methanol as the internal standard ($\Delta\delta_{MeOH}$ = 50.15 ppm).

RESULTS AND DISCUSSION

Most of the strains of marine bacteria producing alginate lyases are closely associated with algae and mollusks [6]. The marine proteobacterium *P. citrea* KMM 3297 isolated from the coelomic liquid of the holothurian *Apostichopus japonicus* exhibits alginolytic properties [10]. In the previous work, we showed that while cultivating on a standard nutrient medium, the bacterium synthesized highly active alginolytic enzymes [11].

It is known that alginic acid is a good inducer of alginate lyases in marine bacteria, while fucoidan as an inducer of alginate lyases has not been studied. To clarify the factors affecting biosynthesis of alginolytic enzymes in *P. citrea* KMM 3297, the dynamics of bacterial growth and the consumption of the components of different nutrient media were investigated. We used glucose (medium A), xylose (medium B), and fucoidan containing about 13% alginic acid (medium C) as the source of carbon.

The composition of the nutrient media was changing during the growth of the bacterial culture. In the case of the glucose-containing medium, the steady-state phase was reached after 18 h, when glucose and other components of the medium were almost completely exhausted (Fig. 1a). In the presence of fucoidan, intensive growth of the bacteria was observed (Fig. 1c), but the stage of cell lysis started after 40 h. Xylose inhibited growth of the culture (Fig. 1b). Peptone was consumed during the first 3 h of growth. The steady-state phase was virtually absent,

and the culture died out quickly. In the latter two cases, no visible consumption of the components of nutrient mixture (peptone and sugars) was observed. Presumably, the bacterium not only consumes, but also synthesizes extracellular polysaccharides and proteins essential for its vital functions under these conditions.

Intracellular alginolytic enzymes of *Pseudoalteromonas citrea* KMM 3297 were synthesized during different phases of the bacterial growth. The maximal activity of the enzymes was observed both in the middle of the exponential phase and in the steady-state phase of the bacterial growth (24-26 and 37 h), decreasing slightly in the later stages in the beginning of the bacterial lysis (Fig. 2).

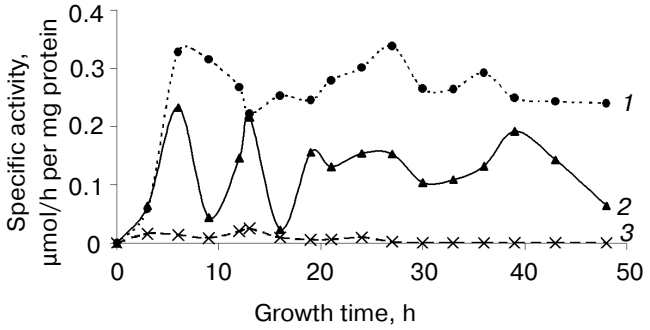

Fig. 2. Alginolytic activity during the growth of the marine bacterium *P. citrea* KMM 3297 in media containing 1 g/liter fucoidan (*1*), 1 g/liter glucose (*2*), and 2 g/liter xylose (*3*).

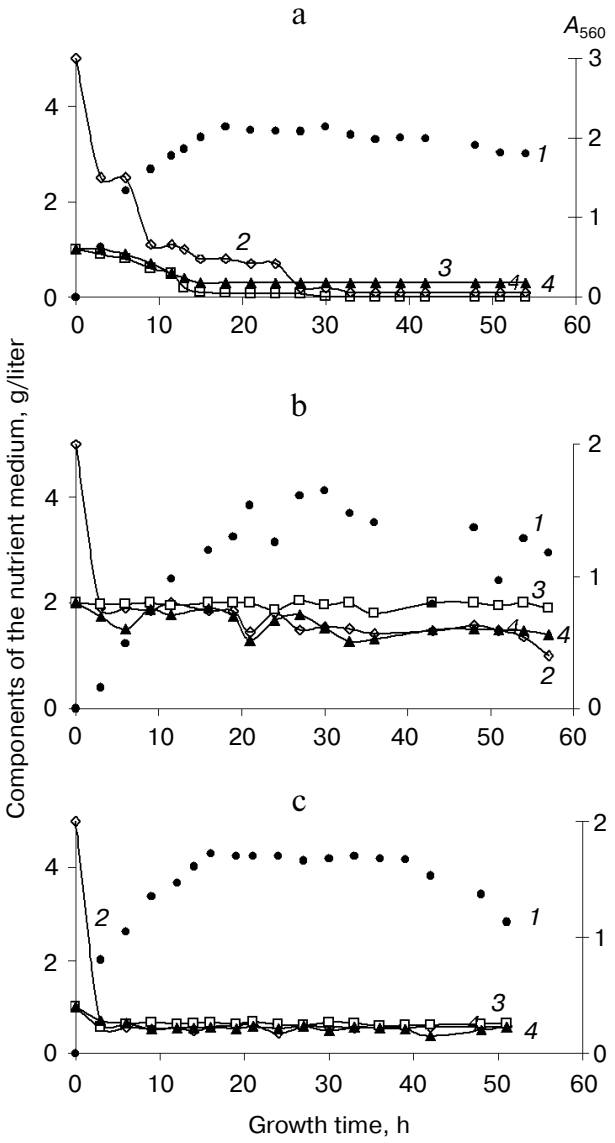

Fig. 1. Growth of the bacterium *P. citrea* KMM 3297 in nutrient media containing glucose (a), xylose (b), and fucoidan (c). *1*) Dynamics of the bacterial growth; *2-4*) consumption of peptone (*2*), total sugar (*3*), and reducing sugars (*4*).

The level of the alginolytic activity depended on the source of carbon. Fucoidan appeared to be an effective inducer of these enzymes (Fig. 2, curve *1*). The optimal range of fucoidan concentration for biosynthesis of the alginolytic enzymes was 1-4 g/liter (Fig. 3). In the presence of glucose, synthesis of the alginolytic enzymes decreased almost twofold (Fig. 2, curve *2*). Xylose was an unfavorable factor for both synthesis of the alginases and bacterial growth (Fig. 2, curve *3* and Fig. 1b, curve *1*, respectively).

The composition of the alginolytic enzymes in the extracts of *P. citrea* KMM 3297 grown in the presence of fucoidan and glucose was analyzed using high-performance anion-exchange chromatography on a Source 15Q PE column. It was shown that in the presence of glucose and peptone, *P. citrea* KMM 3297 produced two forms of the alginolytic enzymes: the main form (AlI) and the minor one (AlII, Fig. 4a). While growing in the presence of fucoidan and peptone, one more isoform of the enzyme appeared (AlIII, Fig. 4b). The appearance of an additional isoform of alginate lyases was observed while growing the marine bacterium *Alteromonas* sp. in a medium containing alginic acid [17].

To isolate the enzymes and to investigate their physical and chemical properties and specificity, the bacterial biomass was grown in the presence of fucoidan. All three forms of the alginases were contained in fraction II (30-70% ammonium sulfate saturation). They were separated on a DEAE-Sepharose CL-6B column using a linear gradient of NaCl (0-0.5 M). The contribution of the separate enzymes AlI, AlII, and AlIII to the total alginolytic activity constituted 52, 4, and 44%, respectively. The fractions containing the alginolytic activity were concentrated by ultrafiltration and then gel filtered. The resulting enzyme preparations had the following specific activities: AlI, 0.4 U/mg protein, AlII, 0.3 U/mg protein, AlIII, 0.4 U/mg protein.

The alginolytic enzymes from *P. citrea* KMM 3297 exhibited maximal activities in the range of pH 7.0-8.0 that are characteristic for the bacterial enzymes, and

retained 100% of activity at 35-45°C at pH 7.2. The molecular weights of the enzymes are 25, 79, and 61 kD for AlI, AlII, and AlIII, respectively. Among marine bacteria there are some species producing two or more molecular forms of alginases that differ in their molecular weights and substrate specificity. The presence of several molecular forms of intracellular alginate lyases, the most part of which is induced by alginic acid, was shown for the marine bacteria of *Alteromonas* genus [17]. The molecular weights of intracellular alginolytic enzymes of marine bacteria known from the literature vary over the wide range from 30 to 100 kD [5].

For comparative study of the properties and the specificity of the alginolytic enzymes AlI, AlII, and AlIII we used two alginic acids as substrates: a commercial preparation of polyguluronic acid and polymannuronic acid isolated from the brown alga *F. evanescens* [12]. Conclusions on the structure of the substrates were based on ^{13}C-NMR spectra of the two polysaccharides. The ^{13}C-NMR spectrum of the commercial alginic acid exhibited six main signals at 102.04 ($J_{C1\text{-}H1}$ = 171.1) (C1), 66.2 (C2), 70.25 (C3), 81.3 (C4), 77.1 (C5), and 177.1 ppm (C6) and minor signals at 101.2-100.77 (C1), 72.06 (C2), 72.6 (C3), 79.07 and 78.6 (C4), 77.1 (C5), and 177.1 ppm (C6). The ^{13}C-NMR spectra of the alginic acid from *F. evanescens* contained the following signals: 101.4 ($J_{C1\text{-}H1}$ = 163) (C1), 71.3 (C2), 72.7 (C3), 79.3 (C4), 77.3 (C5), and 176.6 ppm (C6) and minor signals 102.5 (C1), 66.1 (C2), 81.5 (C4), 68.5 ppm (C5). The spectra of the commercial alginic acid and the alginic acid from *F. evanescens* agree well with the known spectra of polyguluronic and polymannuronic acids, respectively [18]. Thus, the commercial alginic acid is a polysaccharide composed mainly of the residues of guluronic acid (G) connected by α-1,4-O-glycoside bonds also containing a small number of residues of mannuronic acid included into the main chain. Its molecular weight determined by gel filtration was 8-10 kD. Alginic acid isolated from the brown alga *F. evanescens* consists mainly of residues of mannuronic acid (M) connected by β-1,4-O-glycoside bonds. As determined by gel filtration, this alginic acid exhibits polydisperse molecular weight (5-70 kD).

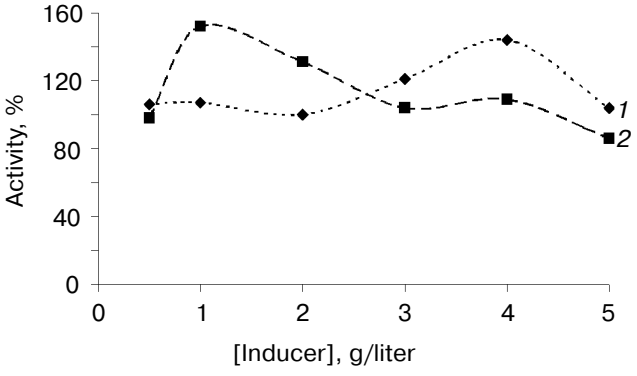

Fig. 3. Effect of different concentrations of fucoidan on the alginolytic activity after 24 h (*1*) and 37 h (*2*) of growth of *P. citrea* KMM 3297. The total alginolytic activity of the extracts of the bacterial grown in the absence of carbohydrates was taken as 100%.

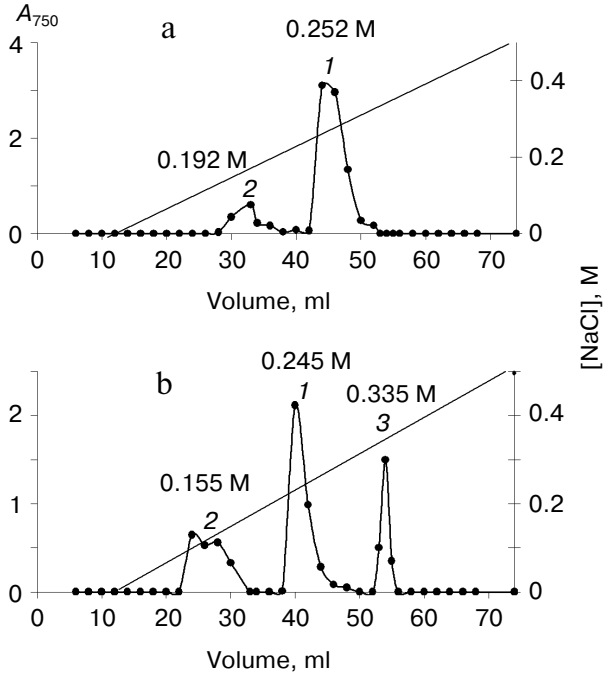

Fig. 4. Chromatography of alginolytic enzymes AlI (*1*), AlII (*2*), and AlIII (*3*) from the marine bacterium *P. citrea* KMM 3297 grown in the presence of glucose (a) and fucoidan (b).

UV spectra of oligouronides obtained after hydrolysis of both alginic acids in the presence of the investigated enzymes exhibited an absorption band at 235 nm. The ^{13}C-NMR spectra of the same products exhibited signals at δ = 145.9, 109.0, and 95 ppm. These signals are characteristic for C4 and C1 of 4-deoxy-L-erythro-hex-4-enopyranosyluronic acid located on the non-reducing terminus of the oligomers of guluronic and mannuronic acids [19] that can be formed by the β-elimination reaction catalyzed by alginolytic enzymes. These facts support the specification of AlI, AlII, and AlIII as alginate lyases.

Final products of degradation of polyguluronate and polymannuronate catalyzed by the alginate lyase AlI were oligouronides with the polymerization degree (n) $5 \geq n \geq 3$ (Fig. 5, a and f); the alginate lyases AlII and AlIII catalyzed degradation of the alginic acids to larger fragments (Fig. 5, (b, g) and (c, h), respectively). The products of the conjoint action of these enzymes were also low-molecular-weight polysaccharides with polymerization degree $5 \geq n \geq 3$ (Fig. 5d). No mono- or disaccharides were observed. Thus, the products of the hydrolysis of polymannuronate and polyguluronate obtained after digestion of the polysaccharides with the investigated alginate lyases characterized these enzymes as endo-type

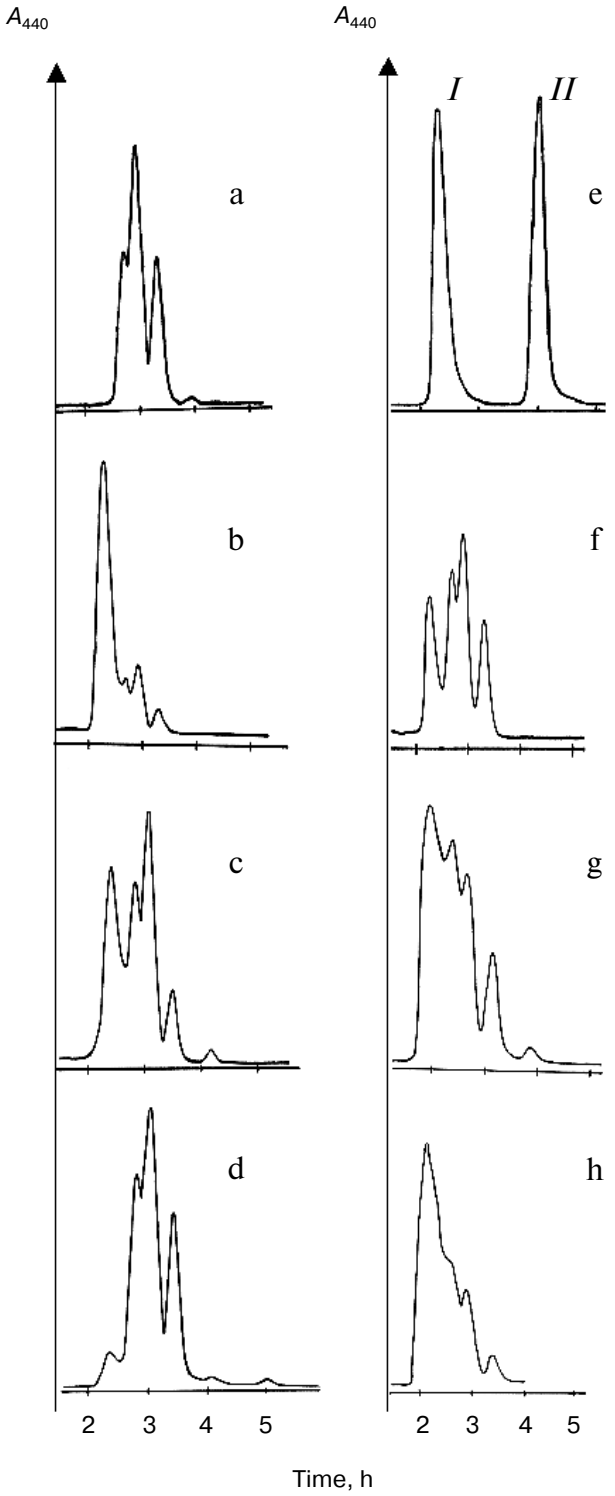

Fig. 5. Biogel P-2 chromatography of the products of hydrolysis of guluronic acid (a, b, c, d) and mannuronic (f, g, h) acids in the presence of the individual alginolytic enzymes AlI (a, f), AlII (b, g), or AlIII (c, h) from *P. citrea* KMM 3297, or in the presence of their mixture (d). Standards: polyguluronic or polymannuronic acids (e, I) and glucuronic acid (e, II).

enzymes, cleaving the internal O-glycoside bonds of the polysaccharide. In the case of exo-type enzyme, we would have observed accumulation of mainly one product (mono-, di-, or trimer), as shown for the alginate lyase from the marine bacterium associated with the brown alga *Sargassum* sp. [20].

The properties of the alginate lyases were investigated by monitoring the enzymatic reaction by increase in absorption at 235 nm (ΔA_{235}) with time τ. The dependences of ΔA_{235} on τ for the elimination reaction of polyguluronic acid catalyzed by AlI, AlII, and AlIII are presented in Fig. 6. The values of the initial rates of the reaction (v, ΔA_{235}/min) determined from these dependences are 0.0052, 0.0010, and 0.0015 for the alginate lyases AlI, AlII, and AlIII (Fig. 6, curves *1*, *2*, and *3*, respectively) and 0.0124 for the mixture composed of equal volumes of these enzymes (Fig. 6, curve *4*). It is seen that the mixture of three enzymes hydrolyses the commercial preparation of alginic acid containing mainly polyguluronic blocks with the rate that ~1.6-fold higher than the theoretical value ($v_I + v_{II} + v_{III} = 0.0077$ against 0.0124 (Fig. 6, curve *5*)). Thus, investigating the mixture of enzymes of the alginolytic complex, we observed a synergistic effect of sequentially–parallel action of three alginate lyases on the polymeric substrate. Presumably, the presence of all three enzymes is necessary for the efficient hydrolysis of alginic acid. A similar effect was observed for the joint action of two alginate lyases ALV and ALVII from *Pseudomonas* sp. OS-ALG-9 on polymannuronic acid [21].

Determination of the Michaelis constants (K_m) for the alginate lyases AlI and AlIII towards polyguluronic

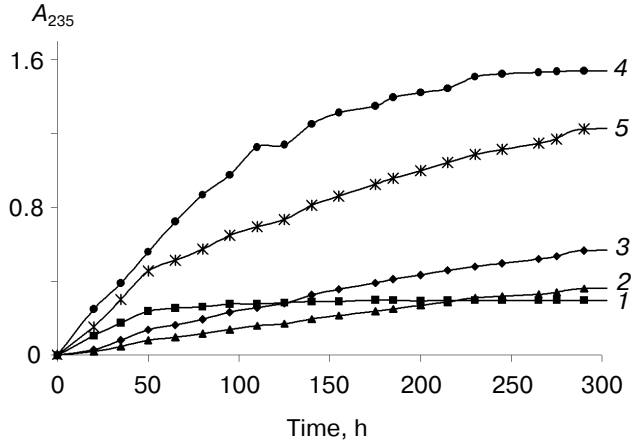

Fig. 6. Dependence of ΔA_{235} on time of incubation: polyguluronic acid (1 mg/ml) was incubated in 0.1 M phosphate buffer, pH 7.2, at 20°C in the presence of AlI (*1*), AlII (*2*), AlIII (*3*), or in the presence of the mixture composed of equal volumes of these enzymes (*4*); theoretical curve for the enzyme mixture (*5*).

and polymannuronic acids showed that both alginate lyases are likely to be enzymes of wide specificity. Alginate lyase AlI exhibits virtually the same affinity to poly-G and poly-M blocks (24 and 34 μg/ml, respectively). The alginate lyase AlIII prefers poly-G blocks (40.0 μg/ml) to poly-M (130.0 μg/ml). Many alginate lyases from gram-negative bacteria exhibit specificity only to poly-M or to poly-G [5]. Alginate lyases of the mixed GM- or MG-specificity have been found in the strains of the marine bacteria *n* 8 and 9 [22, 23], and in *Alteromonas* H-4 identified later as *Pseudoalteromonas elyakovii* [3]. The latter is a relative of the investigated *Pseudoalteromonas citrea* in terms of their phylogenetic properties [24].

The table presents data on the effect of Na^+ and bivalent metal cations on the activity of the alginate lyases AlI, AlII, and AlIII towards polyguluronic acid as the substrate. NaCl (0.2 M) increased the activity of all the investigated enzymes virtually twofold compared to the standard conditions. Presumably, high ionic strength is necessary for the investigated enzymes, as for most alginate lyases from marine bacteria known from the literature [5, 25]. For some of them optimal concentration of NaCl have been determined. This group of enzymes includes alginate lyases from the marine bacterium ATCC 433367 [20, 26].

The salts $MgCl_2$ and $MgSO_4$ (0.01 M) increased the activity of all the investigated enzymes more than twofold (table). Similar results were obtained for the poly-GM-specific alginate lyase from *Alteromonas* H-4 [4]. It should be noted that the activity of many bacterial alginate lyases exhibiting specificity to poly-G blocks of polyuronic acids also increases in the presence of Mg^{2+} [5].

EDTA and Ca^{2+} did not affect the activity of the alginate lyase AlI, and Mn^{2+} inhibited the enzymes AlII and AlIII (table).

The effect of bivalent metal cations on the enzyme activity is closely connected with their specificity. Many of poly-M-specific alginate lyases of bacterial or animal origin do not require Ca^{2+} [5]. A stimulating effect of Ca^{2+} is characteristic mainly for poly-G-specific alginate lyases. For example, 0.1 M $CaCl_2$ increased eightfold the activity of the polyguluronate lyase from the F-6 strain of the bacterium *Pseudomonas* sp. [27]. The enzyme isolated from *Clostridium alginolyticum* was activated by $CaCl_2$ in the concentration range of 0.1-7 mM, but the subsequent increase in Ca^{2+} concentration resulted in transformation of alginic acid into a gel that probably hampered the enzyme action [6]. It is thought that Ca^{2+} bound to poly-G blocks of alginic acid enhance the interaction of the carboxyl ion at C6 with the nucleophilic groups of the active site of the enzyme [28].

The inhibiting effect of Mn^{2+} as well as other metal ions (Zn^{2+}, Cd^{2+}, Hg^{2+}) has been demonstrated for many alginolytic enzymes of different specificity [6] including the poly-M-specific alginate lyase ALVII from *Pseudomonas* sp. OS-ALG-9 [21].

Thus, the present investigation demonstrated that the alginolytic properties of the marine bacterium *P. citrea* KMM 3297 are due to the presence of at least three intracellular alginate lyases (we are not considering extracellular enzymes in this work). Biosynthesis of the intracellular alginate lyases depends on sources of carbon that are the components of the nutrient medium. Fucoidan is an inducer, while xylose inhibits synthesis of these enzymes virtually completely. The three alginate lyases are similar in their physical and chemical properties (pH optima, thermal stability, effect of bivalent metal ions), but differ in their molecular weights and substrate specificity. All these alginate lyases are endo-type enzymes and catalyze degradation of alginic acid yielding polyuronides

Effect of bivalent metal salts and NaCl on the activity of alginolytic enzymes from the marine bacterium *P. citrea* KMM 3297

Ion	Ion concentration, M	Activity, %		
		AlI	AlII	AlIII
Control	0	100	100	100
EDTA	0.01	93	н.о.	н.о.
NaCl	0.2	205	240	202
$MgCl_2$	0.01	205	215	223
$MgSO_4$	0.01	220	215	224
$CaCl_2$	0.01	102	125	97
$MnCl_2$	0.02	n.d.	26	24
NaCl + $MgSO_4$	0.02	195	232	205
NaCl + $MgCl_2$		194	199	211

Note: Control is enzyme activity under standard conditions; n.d., not determined.

of polymerization degree no less than 3. The joint action of these enzymes on the polymeric substrate is more efficient. The ability of the bacterium to synthesize alginolytic enzymes of different specificity and to regulate the composition of the alginolytic enzymes depending on the source of nutrition provides flexibility of the microbial culture in its adaptation to the environment and successful utilization of alginic acids of brown algae by the bacterium *P. citrea* KMM 3297.

The microbiological part of this work was supported by the Russian Foundation for Basic Research (grant No. 02-04-49517) and by the Ministry of Science and Industry of Russian Federation (grant No. 03-19). The chemical part of this work was supported by the Russian Foundation for Basic Research (grant No. 03-04-49534).

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