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Characterization of structural component of cell walls of alkalophilic strain of *Bacillus* sp. C-125

Preparation of poly(γ-L-glutamate) from cell wall component

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The cell wall of an alkalophilic strain of *Bacillus* sp. C-125 is composed of A1γ-peptidoglycan, a teichuronic acid and an unknown acidic polymer composed of glutamic acid and glucuronic acid, of which the molar ratio is approx. 4–5:1. Poly(γ-L-glutamate) was prepared from the acidic polymer by removal of almost all of the glucuronic residues with trifluoromethanesulphonic acid treatment and purified chromatographically. The M_r of the polyglutamate preparation was estimated to be 14000 by gel chromatography, or 43000 on the basis of the content of *N*-terminal acid residues. The acidic polymer found in the cell wall of the organism was concluded to be a polyglutamate substituted with (oligo)glucuronic acid residues or a complex composed of two kinds of polymers (polyglutamate and polyglucuronate).

INTRODUCTION

Cell walls of the Gram-positive bacteria are known to consist of the basal structure, peptidoglycan, and some of the specific polymers such as proteins and polysaccharides. Also, cell walls of alkalophilic strains of *Bacillus* are composed of A1γ type of peptidoglycan and acidic polymers specific for strains. However, our knowledge of the acidic polymers is very poor. The amounts of the acidic compounds (glutamic acid, aspartic acid, glucuronic acid and galacturonic acid) found in group 2 strains are enhanced when the strains are cultured at an alkaline pH (Aono & Horikoshi, 1983; Aono *et al.*, 1984). The acidic polymers extracted from *Bacillus* sp. strain C-125 (classified in the group 2) were separated chromatographically into two fractions. One is a teichuronic acid. The other is an unknown polymer composed predominantly of glutamic acid and glucuronic acid (Aono, 1985; Aono & Uramoto, 1986).

Previously, the author pointed out the biological interest of the unknown polymer. The negative charges on the acidic polymer and a teichuronic acid enclosing the cell wall peptidoglycan of the organism should give the cell surface its abilities to adsorb sodium and hydronium ions and repulse hydroxide ion, and as a result, could enable the cells to grow in an alkaline environment (Aono, 1985). The chemical nature of the polymer, however, was not clearly concluded.

The present paper presents data on the preparation and characterization of the polyglutamate freed from glucuronic acid.

MATERIALS AND METHODS

Organism and isolation of non-peptidoglycan component from cell wall

The alkalophilic strain of *Bacillus* sp. C-125 (Honda *et al.*, 1985) was cultured at an alkaline pH. Cell walls were prepared from the organism in the early stationary phase of growth by inactivation of autolytic enzymes with SDS at 60 °C, disruption with a sonic oscillator and tryptic digestion, as described previously (Aono & Horikoshi, 1983). Non-peptidoglycan components were extracted with 5% (w/v) trichloroacetic acid and fractionated by DEAE-cellulose column chromatography. The acidic substance composed of glucuronic acid and glutamic acid was recovered. The substance prepared from the cells grown at pH 10 is referred to as 'A2 substance' (Aono, 1985).

TFMS treatment of the A2 substance

The A2 substance (1 mg) was placed in glass tubes and dried *in vacuo* over solid NaOH at 42 °C. To each of the samples, 0.3 ml of a mixture of methoxybenzene and TFMS (1:2, v/v) was added (Edge *et al.*, 1981). They were incubated at 0 or 25 °C. Periodically, one of the reaction mixtures was refrigerated at −20 °C overnight, after addition of 6 ml of 10% (v/v) hexane in diethyl ether. Precipitates were recovered by centrifugation at 2500 *g* for 10 min at room temperature and washed three times with the hexane/diethyl ether and finally with diethyl ether. They were dried in air, dissolved in 0.4 ml of 0.2 M-NaH_2PO_4/NaOH buffer (pH 7.2) and dialysed against distilled water.

Preparation of polyglutamate chain

The dry A2 substance (401 mg) was suspended in 8 ml of methoxybenzene. To the suspension, cooled in an ice/water bath, 16 ml of TFMS was added. Nitrogen gas was substituted for air in the vessel by repeatedly flushing with N_2. The suspension was shaken in the ice/water bath for 5 h and then at 22 °C for 2 h. A homogeneous solution obtained by the treatments was added dropwise to 100 ml of 10% (v/v) hexane in diethyl ether refrigerated at −20 °C. White insoluble matter formed in the liquid was immediately collected by centrifugation at 2500 *g* for 5 min. The precipitate was washed by repeated centrifugation from suspensions in

Abbreviation used: TFMS, trifluoromethanesulphonic acid.

the hexane/diethyl ether, until acidic substances could not be detected in the supernatant liquid. The product was suspended in 3 ml of distilled water. The residual diethyl ether was removed by a stream of air. The product was dissolved by the addition of pyridine.

The solution was diluted with 5 ml of 0.2 M-acetic acid/pyridine buffer (pH 5.0) and fractionated on a column (2.6 cm × 97 cm) of Sephadex G-15 which had been equilibrated with the same buffer. The fractions containing polyglutamic acid, eluted at the void volume, were dialysed against distilled water. The non-diffusible fraction was purified by a DEAE-cellulose column chromatography. Fractions (12 ml) containing polyglutamate were dialysed against distilled water and freeze-dried. The residual matter was dissolved in 4 ml of 0.3 M-NaCl and applied to a column (2.5 cm × 91 cm) of Sephadex G-75. The column was eluted with 0.3 M-NaCl at a flow rate of 12 ml/h. Fractions (5 ml) containing polyglutamate were collected, dialysed exhaustively against distilled water and freeze-dried.

Partial acid hydrolysis of the polyglutamate preparation and fractionation of oligomeric glutamic acid

The polyglutamate preparation (10 mg dry weight) was partially hydrolysed in 1.2 ml of 1 M-HCl at 100 °C for 1 h. After removal of the HCl *in vacuo* over solid NaOH at 42 °C, the residue was dissolved in distilled water and developed three times on Avicel thin-layer plates in solvent A (see below). Oligomeric compounds were located on a guide strip by ninhydrin spray and extracted with distilled water. The extracts were further purified by fractionation on Avicel thin-layer plates in solvent B (see below).

γ-Glutamyl transferase treatment

The glutamic acid oligomers (0.2 μmol as *N*-terminal residues) were dissolved in 20 μl of 50 mM-NaH_2PO_4/NaOH buffer (pH 7.2) containing 1 mM-$MgSO_4$. To the solutions, 0.01 unit of γ-glutamyl transferase and 0.2 μmol of leucine were added (Hanes & Hird, 1952). After incubation at 35 °C overnight, the reaction was stopped by addition of 40 μl of ethanol. The mixture was centrifuged at 15000 *g* for 5 min at room temperature. The supernatants were recovered and evaporated to dryness *in vacuo*. The residue was analysed by Avicel t.l.c. in solvents A and D (see below).

To investigate if the *N*-terminal residue of the polyglutamate domain was free, 0.2 mg of the sample was incubated with 0.3 unit of the enzyme in 20 μl of the same buffer containing 0.04% NaN_3 without addition of leucine at 30 °C for 1 day (Shaw & Strømme, 1983).

Chemical analysis

Amino acids and amino sugars. Samples were hydrolysed in 4 M-HCl in sealed tubes at 105 °C for 15 h. After removal of HCl, the residues were analysed with an automatic amino acid analyser. L-Glutamic acid was determined with L-glutamate dehydrogenase (Bernt & Bergmeyer, 1974; Beutler & Michal, 1974). Amino sugars were also determined by the Elson–Morgan or Morgan–Elson reactions (Spiro, 1966; Wheat, 1966) with glucosamine as a reference, after hydrolysis in 4 M-HCl at 100 °C for 5 h.

Uronic acid. Glucuronic acid was directly determined by the carbazole/H_2SO_4 reaction (Spiro, 1966).

***N*-Terminal amino acid residue.** The sample was dinitrophenylated and then partially hydrolysed in 3 M-HCl at 100 °C for 15 min. The *N*-terminal amino acid residue was determined by absorbance of the solution at 420 nm (Ghuysen *et al.*, 1966), using glutamic acid as a reference. The dinitrophenylated product was completely hydrolysed in 6 M-HCl at 100 °C for 16 h. The dinitrophenyl amino acid was extracted with diethyl ether.

Miscellaneous compounds. Acetic acid was determined by using an assay kit from Boehringer (Aono, 1985). Sodium ion was determined with an atomic absorption spectrometer (Aono, 1985). Phosphorus was determined after ashing with magnesium nitrate (Ames, 1966).

Molecular mass. M_r values for the samples were estimated by gel chromatography on two columns of Shodex WS 802.5F (Showa Denko, Japan) connected in series, which were equipped to a h.p.l.c. apparatus. The columns were eluted with 0.1 M-NaH_2PO_4/NaOH buffer (pH 7.2) at a flow rate of 1.0 ml/min. The elution pattern was monitored by measuring absorbance at 205 nm with a spectrophotometric detector. M_r standards of pullulans (Shodex Standard P-82; Showa Denko, Japan) were used to calibrate the gel chromatography. The elution patterns of the pullulans were followed by the phenol/H_2SO_4 reaction (Spiro, 1966).

Thin-layer chromatography

Ascending chromatograms were run on Avicel cellulose or silica gel thin-layer plates at room temperature in the following solvents: (A) ethyl acetate/pyridine/water/acetic acid (5:5:3:1, by vol.); (B) ethyl acetate/water/acetic acid (10:6:5, by vol.); (C) butan-1-ol/pyridine/water (6:4:3, by vol.); (D) propan-1-ol/water (4:1, v/v); (E) benzyl alcohol/chloroform/methanol/4 M-NH_4OH (15:15:15:8, by vol.). The components of the samples were located with alkaline silver nitrate or ninhydrin spray.

Materials

TFMS was purchased from Wako Pure Chemical, Osaka, Japan. Dinitrofluorobenzene, pepsin (EC 3.4.23.1) and trypsin (EC 3.4.21.4) were obtained from Sigma. γ-Glutamyl transferase (EC 2.3.2.2) was a product of Oriental, Tokyo, Japan. L-Glutamate dehydrogenase (EC 1.4.1.3) and Pronase E were obtained from Boehringer. DEAE-cellulose was DE 52 grade from Whatman.

RESULTS

Dissociation of the A2 substance with TFMS

A time and temperature study was carried out to examine the effects of TFMS on the release of glucuronic acid residues and on the recovery of intact polyglutamic acid chains (Table 1). The A2 substance was hard to dissolve in the reagent at 0 °C but it had dissolved completely after 5 h. Almost all of the glutamic acid residues were recovered even after the prolonged incubation. An increase in the content of *N*-terminal residue was negligible (results not shown), indicating that peptide bond cleavage had not taken place to a detectable extent. On the other hand, the substance was comparatively easily dissolved in the reagent at 25 °C.

Table 1. Recoveries of components of macromolecules obtained by TFMS treatment

The A2 substance (1 mg) was incubated in 66% (v/v) TFMS solution at 0 or 25 °C. Products were precipitated by the addition of 10% (v/v) hexane in diethyl ether followed by refrigeration at −20 °C. The deglycosylated products were dialysed against distilled water. The non-diffusible material was assayed for the main components found in the A2 substance. Each determination is represented as μmol, and recoveries are given in parentheses. They were calculated taking the contents in the non-diffusible fraction of the substance without TFMS treatment as 100%. The M_r value was determined for the main peak on the gel chromatography as shown in Fig. 4.

Treatment temperature (°C)	Incubation time (h)	Recoveries [μmol (%)]			$10^{-3} \times M_r$
		Glutamic acid	Glucuronic acid	Hexosamines	
0	1	3.61 (100)	0.680 (103)	0.072 (107)	20.0
	2	3.39 (94)	0.633 (95)	0.067 (100)	19.4
	3	3.43 (95)	0.471 (71)	0.054 (80)	18.8
	5	3.73 (103)	0.458 (69)	0.047 (70)	16.5
	10	2.91 (81)	0.244 (37)	0.029 (43)	12.8
	20	2.83 (78)	0.070 (11)	0.009 (14)	12.0
25	1	3.29 (91)	0.129 (19)	0.014 (21)	12.0
	2	2.60 (72)	0.085 (13)	0.010 (14)	11.2
	3	2.41 (67)	0.020 (3)	0.007 (10)	10.6
	5	2.09 (58)	0.020 (3)	0.007 (11)	9.6
	7	1.62 (45)	0.017 (3)	0.008 (11)	8.6
	10	0.53 (15)	0.007 (1)	0.006 (9)	7.4

The recovery of glucuronic acid was dramatically decreased. However, *N*-terminal amino acid residues increased (results not shown) and the recovery of the macromolecular glutamic acid residues decreased. The sizes of the macromolecules obtained under the conditions were considerably lowered, depending on the incubation time. It was concluded that the reagent caused a partial cleavage of peptidyl linkages in a polyglutamate chain. Therefore, I concluded that it would be very hard to selectively remove all the glucuronic acid residues and retain the domain of glutamic acid residues completely intact, but it would be comparatively easy to prepare the polymer composed essentially of glutamic acid, although the preparation might be a partial fragmentation product.

Preparation and purification of polyglutamate from the A2 substance with TFMS

A large amount of the A2 substance (401 mg dry weight; 2210 μmol of glutamic acid; 380 μmol of glucuronic acid) was dissolved gently in the TFMS reagent at 0 °C to avoid non-uniform removal during the dissolution. The substance dissolved after 5 h. The reaction mixture was incubated further at 22 °C for 2 h in order to remove glucuronate residues effectively, because the removal of glucuronic acid was not complete at 0 °C, as shown in Table 1. The polyglutamate precipitated from the reaction mixture was converted to its pyridinium salt and dissolved in distilled water.

The product did not contain any oligomeric glutamic acid, although a trace proportion of glucuronic acid remained (Fig. 1). All of the product was eluted at the void volume of the Sephadex G-15 column. Recovery of glutamic acid in the void volume was 51% of the original A2 substance and that of glucuronic acid was only 1%. This polymer fraction was fractionated by DEAE-cellulose column chromatography (Fig. 2). Glutamic acid residues were eluted as a single peak concomitant with a small amount of glucuronic acid residues. The fractions (yield: 250 mg dry weight, 1335 μmol of glutamic acid, 5.0 μmol of glucuronic acid) were further purified by gel chromatography on a Sephadex G-75 column (Fig. 3). Polyglutamate containing a small amount of glucuronic acid was fractionated also on this column. The yield of the purified product was: 194 mg

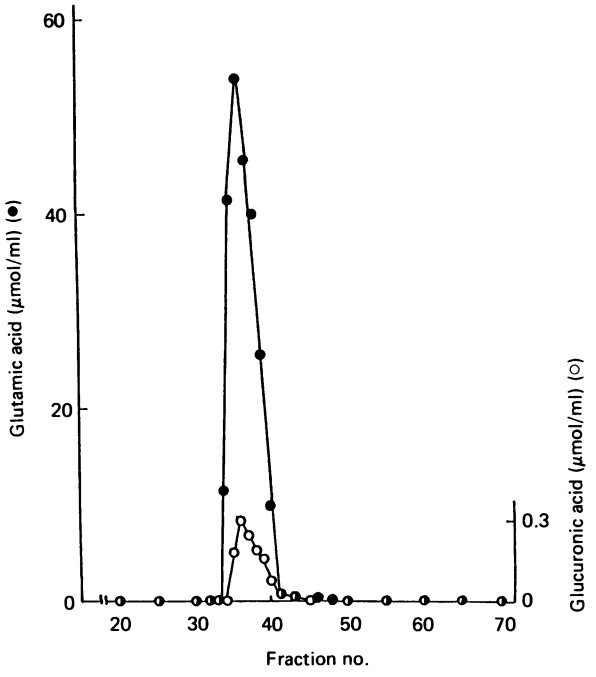

Fig. 1. Sephadex G-15 column chromatography of the A2 substance after treatment with TFMS

The A2 substance (401 mg) was deglycosylated with 16 ml of TFMS reagent. The product was washed with 10% (v/v) hexane in diethyl ether and chromatographed on a column (2.6 cm × 97 cm) of Sephadex G-15 in 0.2 M-acetic acid/pyridine (pH 5.0) at a flow rate of 30 ml/h. Fractions (5 ml) were collected and assayed for L-glutamic acid (●) and glucuronic acid (○). Fractions 34–40 were recovered.

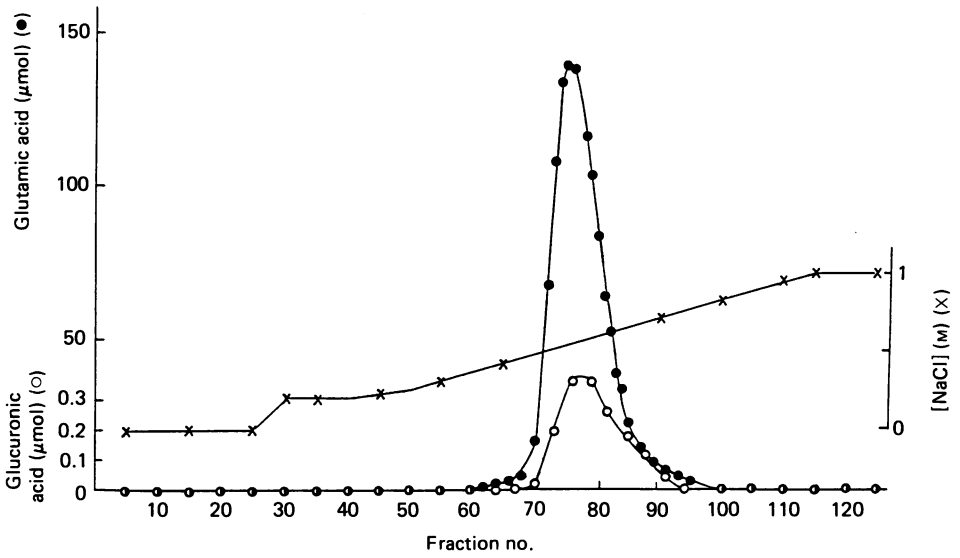

Fig. 2. DEAE-cellulose chromatography of the polyglutamate fraction

The non-diffusible polyglutamate fraction eluted at the void volume of the Sephadex G-15 column was dissolved in 0.05 M-acetic acid/NaOH (pH 5.5) and loaded on a column (2.6 cm × 51 cm) of DEAE-cellulose equilibrated with the same buffer. The column was washed with 100 ml of the same buffer and then with 200 ml of the buffer containing 0.2 M-NaCl at a flow rate of 60 ml/h. Thereafter, the column was eluted at the same rate with a linear gradient from 0.2 to 1.0 M-NaCl in the buffer (500 ml) and then with 200 ml of the buffer containing 1 M-NaCl. Fractions (12 ml) were collected and assayed for L-glutamic acid (●) and glucuronic acid (○). Fractions 69–89 were recovered. Concentration of NaCl (×) was determined by measurement of refractive index of the fractions.

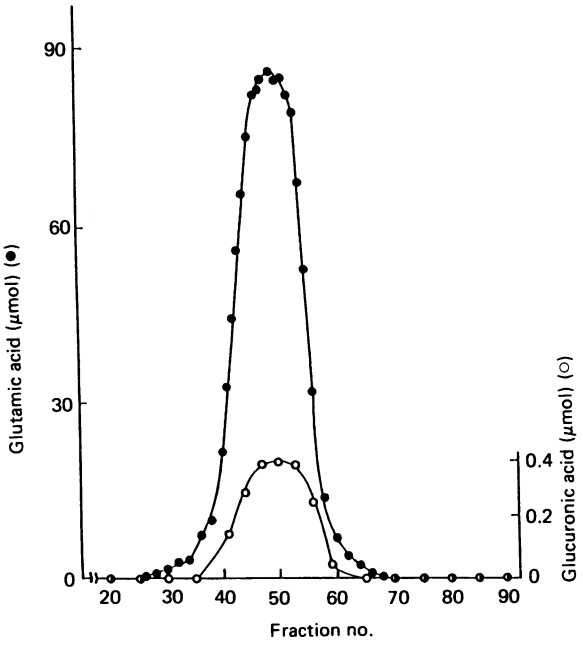

Fig. 3. Elution of the polyglutamate preparation through a column of Sephadex G-75

The polyglutamate fraction from the DEAE-cellulose column was dissolved in 0.3 M-NaCl and chromatographed on a column (2.5 cm × 91 cm) of Sephadex G-75 equilibrated with 0.3 M-NaCl. Fractions (5 ml) were collected and assayed for L-glutamic acid (●) and glucuronic acid (○). Fractions 37–60 were recovered.

dry weight, 1201 μmol of glutamic acid, 4 μmol of glucuronic acid.

The small quantities of glucuronic acid and galactosamine were not removed by the TFMS treatment. Their amounts were respectively about 1 and 7% of those initially found in the substance. The polyglutamate preparation could not be freed from these residues by further purification procedures (Figs. 1–3 and Table 2).

Chemical composition of the polyglutamic acid

The chemical composition of the preparation is shown in Table 2. The A2 substance consists of predominantly glutamic acid, glucuronic acid and sodium. The main components of the polyglutamate preparation were glutamic acid and sodium. Glutamic acid accounted for about 92% of the dry weight of the preparation, assuming the acid residues were present as monosodium salts. These analyses certified the preparation to be almost entirely a polyglutamate.

The M_r of the polyglutamate preparation was estimated by gel chromatography to be 14000 (Fig. 4). On the other hand, the M_r of the original A2 substance was concluded to be 21000.

Characterization of the peptidyl linkage between glutamic acid residues

Four fractions of oligomeric glutamic acid were prepared from the partial hydrolysate of the polyglutamate preparation. Each of the fractions was incubated with γ-glutamyl transferase and then analysed on Avicel t.l.c. plates in solvents A and D. The spots of the original oligomers were diminished and monomeric

Table 2. Chemical composition of the A2 substance and the polyglutamate preparation purified by DEAE-cellulose and gel chromatography

The experimental details are described in the Materials and methods section. Values are μmol/mg. Molar ratios (given in parentheses) were calculated taking L-glutamic acid as 1.0. Abbreviations: –, not tested; N.D., not detected.

Component	Composition [μmol/mg (molar ratio)] of: A2 substance	Polyglutamate
L-Glutamic acid	4.4 (1.0)	6.1 (1.0)
Glucuronic acid	0.96 (0.22)	0.015 (0.0024)
Galactosamine	0.039 (0.0089)	0.0041 (0.00067)
Glucosamine	0.0086 (0.0020)	N.D.
Fucosamine	0.0044 (0.0010)	0.0031 (0.00051)
Acetic acid	0.097 (0.022)	–
Sodium	4.2 (0.95)	4.9 (0.80)
Phosphate	0.0009 (0.0002)	–
Muramic acid	0.0055 (0.0013)	0.0010 (0.00016)

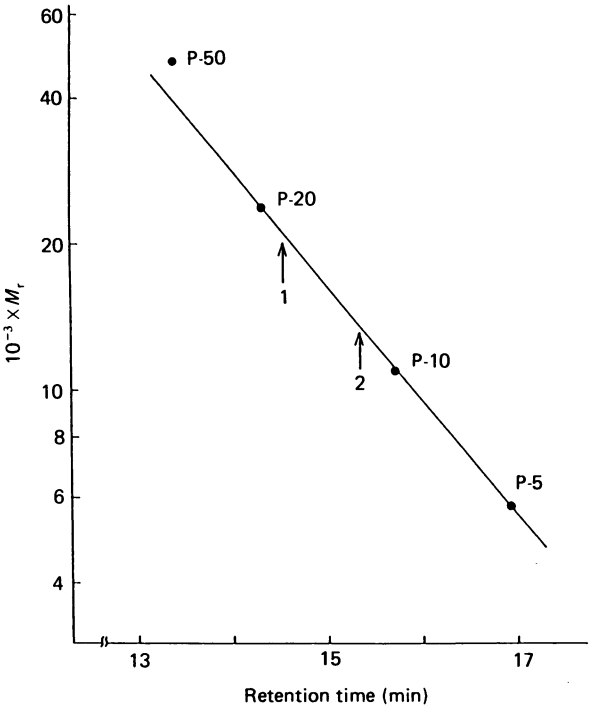

Fig. 4. Determination of M_r values of the A2 substance and polyglutamate preparation

The M_r values of the samples were measured by gel chromatography on Shodex WS 802.5F columns. The graph shows the retention times of: the A2 substance (1), the polyglutamate preparation (2), and pullulans P-50, P-20, P-10 and P-5.

glutamic acid was released in all the fractions examined. These results showed that the glutamic acid residues were bound to one another through γ-peptidyl linkages, not through α-linkages, in all the oligomers. Therefore, the preparation was concluded to be a sodium salt of poly(γ-L-glutamic acid). The sizes of the polyglutamate preparation and the A2 substance were not altered by incubation with several proteolytic enzymes of various substrate specificities, for example, trypsin, pepsin and Pronase E, as far as examined by gel chromatography (results not shown). This result supported the conclusion that the glutamic acid residues did not bind together through α-peptidyl linkages.

Identification and determination of *N*-terminal amino acid residue

The *N*-terminal amino acid in the A2 substance and the polyglutamate preparation was dinitrophenylated. After the complete hydrolysis of the dinitrophenylated products, the dinitrophenyl compounds were analysed by Avicel t.l.c. in solvent C and by silica gel t.l.c. in solvent E. Only dinitrophenyl-glutamic acid was found in the hydrolysates from the samples. The content of the terminal residue was 23.4 nmol/mg in the polyglutamate preparation and 11.5 nmol/mg in the A2 substance.

Characterization of the state (free or esterified) of the terminal carboxyl group

The hydrolysis reaction by γ-glutamyl transferase was utilized to characterize whether the α-carboxyl group of *N*-terminal glutamic acid residue was free or not. The polyglutamate preparation and the A2 substance (respectively 4.7 and 2.3 nmol as *N*-terminal glutamic acid) were incubated with a large amount of γ-glutamyl transferase without an acceptor molecule for a long time. Monomeric glutamic acid was found by Avicel t.l.c. to be released from the samples. The amounts of free glutamic acid determined with L-glutamate dehydrogenase were respectively 7.5 and 5.7 nmol/nmol of *N*-terminal glutamic acid residue found in the polyglutamate preparation and the A2 substance. These results suggested that α-carboxyl group of the *N*-terminal glutamic acid residue was free.

DISCUSSION

So far, the fraction A2 substance could not be separated into two fractions containing only either polyglutamic acid or polyglucuronic acid when examined with any chromatographic separation. Failure of the attempt supported superficially the possibility that the substance is a copolymer of glutamic acid and glucuronic acid. An attempt to prepare a putative repeating unit, namely an oligomer composed of a few residues of glutamic acid and glucuronic acid, from the partial acidic hydrolysates of the substance under various conditions was unsuccessful. This failure superficially suggested that the two acid residues did not bind to each other as a repeating unit. A method to deglycosylate glycoproteins with TFMS was applied to the substance to prepare the polyglutamate chain region.

Optimum conditions for removal of glucuronic acid residues were first worked out by a time and temperature study. The A2 substance was treated with the reagent under the mildest possible conditions. Under these conditions, a long reaction time was required because of the insolubility of the substance in the reagent. The treatment might cause heterogeneous removal of the glucuronic acid residues and partial cleavage in the polyglutamate domain. The M_r value of the polyglutamate preparation was estimated to be 14000 by Shodex WS 802.5F gel chromatography using pullulans as M_r references (Fig. 4). The M_r of the A2 substance was

21000 in this system. Previously, I reported that the size of the A2 substance was 19000 by Toyopearl HW55S gel chromatography using dextrans as M_r references (Aono, 1985). These values, however, might not be correct, because the M_r references used were chemical digestions of pullulan and dextran which were neutral polyglucans. The former is a roughly linear molecule and the latter is a roughly spherical one. The molecular forms of the samples were not known, although I assumed that the form of the A2 substance should be linear (Aono, 1985). Other factors such as ion exclusion and hydration effects must also be considered, because all the samples were highly negatively charged with a great number of carboxyl groups.

On the assumption that only one *N*-terminal glutamic acid residue should exist in every molecule, the M_r of the polyglutamate preparation was calculated to be 43000 on the basis of *N*-terminal residue content. The M_r of the original A2 substance was estimated to be 87000. Considering the *N*-terminal glutamic acid determination and total glutamic acid content in the preparation, a chain of polyglutamate contains on average 262 molecules of glutamic acid. The M_r of the sodium salt of the polyglutamate was deduced to be 40000 from the number of glutamic acid residues. The comparison between the size of the polyglutamate preparation estimated by the two methods and that of the A2 substance indicates that the glutamic acid residues must form only one block in the A2 substance. If the glutamic acid residues formed two or more blocks, the M_r should be lower than that experimentally determined for the polyglutamate preparation.

Therefore, the substance was concluded to be a complex polymer composed of one chain of poly(γ-L-glutamate). The chain is bound to one polyglucuronate chain or has many (oligo)glucuronate substituents. Characterization of the chemical nature of the glucuronate region is under study. The free *N*-terminal residue was glutamate in the polyglutamate region of the A2 substance, and the α-carboxyl group of this residue was also free. At least the *N*-terminal glutamic acid is not substituted with a glucuronic acid residue. This type of polyglutamate is likely to exist widely in the group 2 strains of alkalophilic *Bacillus*, because large amounts of glutamic acid and glucuronic acid were always found in their cell walls and were extractable with trichloroacetic acid (Aono & Horikoshi, 1983; Aono *et al.*, 1984).

γ-Polyglutamate has been found widely in other bacteria belonging to the genus *Bacillus*, e.g. *B. anthracis*, *B. mesentericus and B. subtilis* (Ivánovics & Bruckner, 1937; Bovarnick, 1942; Hanby & Rydon, 1946; Thorne *et al.*, 1954). These are capsular structures or extracellular mucilaginous materials, and do not bind to the cell wall peptidoglycan layer of the producers. Moreover, they do not contain glucuronic acid as a structural copolymer. The results presented in the present paper show that poly(γ-L-glutamic acid) is a structural component of the cell wall of alkalophilic *Bacillus* C-125 and constitutes a predominant portion of the complex polymer, the A2 substance.

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