Carbohydrate Research, 57 (1977) 163–171

ISOLATION OF A POLY-α-L-GULURONATE LYASE FROM *Klebsiella aerogenes**†

JONATHAN BOYD AND JAMES R. TURVEY

School of Physical and Molecular Sciences, University College of North Wales, Bangor, N. Wales, LL57 2UW (Great Britain)

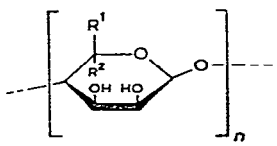
(Received December 3rd, 1976; accepted for publication, December 23rd, 1976)

ABSTRACT

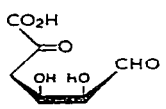
The bacterium *Klebsiella aerogenes* type 25 produces an extracellular alginolyase which has been partly purified. The enzyme is specific for the α-L-guluronosyl linkages in whole alginate and fractions therefrom. The end products of its action on polyguluronic acid blocks are mainly the unsaturated di- and tri-saccharides, with a smaller proportion of the homologous tetrasaccharide. Some general properties of the enzyme are reported.

INTRODUCTION

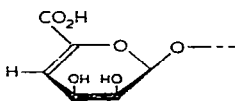
Alginic acid from brown seaweeds consists of a copolymer of three main structural blocks, poly-β-D-mannuronic acid (poly-M, **1**), poly-α-L-guluronic acid (poly-G, **2**), and blocks in which both uronic acids occur in what is believed to be an alternating sequence (poly-MG)[1,2]. The proportion of the various blocks in an alginate sample depends upon the source of the alginate, and varies in different parts of the algal tissue[3]. The poly-G blocks complex strongly with Ca^{2+}, Ba^{2+}, and certain other divalent ions[4,5], leading to alginates which gel readily with these ions; the other blocks complex only weakly and give alginate fractions which form viscous solutions. In order to understand the relationship between gel formation and the block structure of alginates, it is necessary to know the chain lengths of the various blocks and how they are distributed in alginates from various sources.



1 $R^1 = CO_2H$, $R^2 = H$
2 $R^1 = H$, $R^2 = CO_2H$



3



4

*Dedicated to the memory of Sir Edmund Hirst, C.B.E., F.R.S.
†Studies of Alginate-Degrading Enzymes: Part I.

Enzymes which depolymerise alginate have been obtained from various bacteria[6,7], from brown algae[8,9], and from marine molluscs[10,11]. In general, highly purified, bacterial enzymes have shown a preference for cleaving the α-L-guluronic acid linkage, whereas purified enzymes from algae and molluscs have greater activity on the β-D-mannuronic acid linkage. All of these enzymes are lyases, in that they cause cleavage of the glycosidic linkage by elimination, leading to formation of an αβ-unsaturated acid, 4-deoxy-L-*erythro*-hex-5-ulosuronic acid (**3**), at the non-reducing end of the oligosaccharide **4**. This unit, produced by enzyme cleavage, can arise from either a mannuronic or a guluronic acid unit. However, enzymes having known specificity for only one type of linkage could still be used to obtain information on the lengths of the blocks not attacked. We now report the isolation of an enzyme, specific for the α-L-guluronosyl linkage in alginic acid, from a mildly pathogenic organism, *Klebsiella aerogenes* type 25. This enzyme was reported to us as degrading both algal alginic acid and the polysaccharide mixture from a mucoid strain of *Pseudomonas aeruginosa*, the latter mixture probably containing bacterial alginate as one of its components[12].

RESULTS AND DISCUSSION

Growth of the organism on a nutrient–alginate medium suggested that the alginolyase was extracellular, being readily separated from bacterial cells by centrifugation. Enzyme activity in the medium was maximal after 30-h incubation, when bacterial numbers were still increasing (Fig. 1), indicating that the enzyme was not a product of cell lysis. The enzyme was precipitated from the cell-free medium with ammonium sulphate, but polysaccharide was still present in the preparation. This enzyme was eluted as a single peak, together with protein, from Sephadex G-100, but two active fractions were eluted from a column of Sephadex G-150, one at the exclusion limit of the gel and the other as a component of lower molecular weight. The two fractions, however, had identical pH-optima and the same action on various substrates, and it was concluded that the fraction excluded by the gel was an enzyme–polysaccharide complex, while the other fraction represented the free enzyme. Separation of enzyme activity from inactive protein and polysaccharide was best achieved on an ion-exchange gel, DEAE Sephadex A-50, from which the enzyme was eluted as a single peak, free from polysaccharide. The recovery and specific activities at various stages are shown in Table I. The enzyme could not be further fractionated on Sephadex gels.

Properties of the enzyme. — The enzyme displayed a pH optimum at 7.0, and it was stable to freeze-drying, retaining activity for several months in this form. The activity of the enzyme on alginate could be monitored by increase in reducing power, by decrease in viscosity of the alginate, or by production of the unsaturated uronic acid moiety **4**. Formation of **4** was readily followed by measuring the u.v. absorption at 230 nm[7], or by the specific formation of 3-formylpyruvic acid after periodate oxidation, and determination with thiobarbituric acid[13]. The effect of various salts

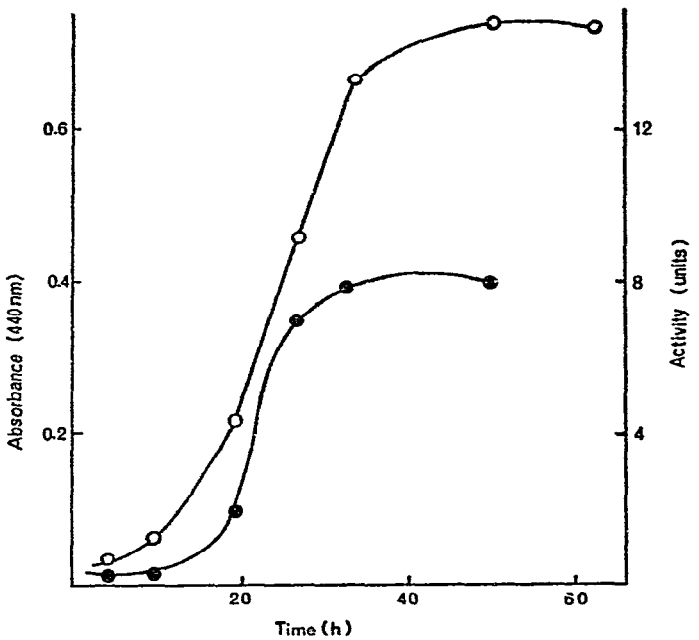


Fig. 1. Growth of organism and production of extracellular enzyme. Bacterial numbers were monitored by absorbance at 440 nm (—○—), and enzymic activity by viscometry (—●—).

TABLE I

PURIFICATION OF ENZYME

Stage	*Activity* (10^4 *units*)	*Protein* (*g*)	*Specific activity* (*units*.mg^{-1})
Culture supernatant	8.37	—	—
$(NH_4)_2SO_4$ precipitate	3.26	2.93	11.1
Dialysed precipitate	3.21	1.46	21.9
Eluted from column	2.55	0.140	181.6

on enzyme activity (Table II) showed that, like other alginolyases[11], monovalent cations produced activation at 0.3M concentration, whereas Mg^{2+} caused activation at lower concentrations (~0.1M), higher concentrations causing lower activity or precipitation of alginate. That the enzyme is an endo-enzyme is shown by a comparison, in the same digest, of the decrease in viscosity of an alginate solution (expressed as the reciprocal of ln η_R) against the increase in u.v. absorbance (Fig. 2). A linear relationship indicates the random cleavage of glycosidic linkages typical of an endo-enzyme[14]. The enzyme had no action on laminarin, dextran, yeast mannan, lichenin, or porphyran, but contained slight amylase activity when tested on amylose and glycogen.

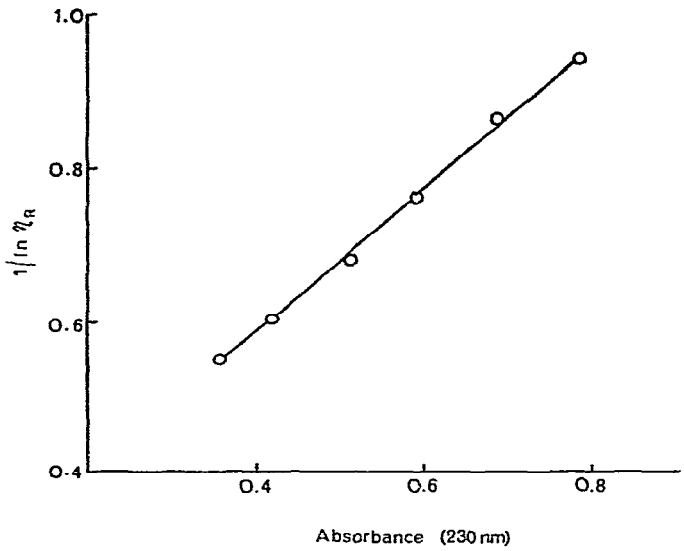


Fig. 2. Relationship between the natural logarithm of the relative viscosity of alginate during enzyme degradation and the number of bonds broken, as given by the production of unsaturated uronic acid (absorbance at 230 nm).

TABLE II

EFFECT OF SALTS ON ENZYME ACTIVITY

Salt	*Activity (absorbance at 540 nm*[a])				
	*0.025*M	*0.05*M	*0.1*M	*0.3*M	M
NaCl	5.6	8.0	8.8	9.3	7.7
KCl	6.3	7.6	7.8	8.1	5.8
KBr	6.2	6.6	7.0	7.2	5.3
$MgCl_2$	6.9	7.7	9.0	8.6	5.6
$MgSO_4$	6.9	8.5	8.3	7.1	—

[a]Absorbance in 1-cm cell with dinitrosalicylate reagent.

Action on alginate and separated blocks. — The percentage degradation (as mannuronic acid) of whole alginates and of various blocks is shown in Table III, in which the degradation is compared with the ratio (M/G) of the two uronic acids in the various substrates. It should be noted that the extent of degradation (as mannuronic acid) was calculated on the assumption of an infinite chain-length (c.l.) for the substrate and that, theoretically, all bonds could be cleaved to give monomer with reducing power equivalent to that of an equimolar amount of mannuronic acid. The result is, therefore, dependent on the initial c.l. of the substrate, and on the fact (see later) that oligomers, rather than monomers, are the end products of enzyme action. Calculation shows that for a poly-G sample of c.l. 15, the maximum reducing-power

increase could be 33%. As can be seen, activity was highest on short-chain poly-G blocks, and G-rich alginate (alginate IV), intermediate with poly-MG blocks, and lowest with M-rich alginates and poly-M blocks. However, a plot of percentage degradation against M/G ratios is not linear, and the method cannot be used to predict M/G ratios with any accuracy. Enzyme action on poly-M blocks was very limited when assayed by u.v. absorption, being complete within 1 h under conditions where poly-G blocks were still being degraded after several hours. This slight degradation of poly-M blocks is probably due to the small content of guluronic acid units (Table III).

TABLE III

COMPARISON OF EXTENT OF ENZYMIC DEGRADATION WITH M/G RATIOS

Sample	*M/G ratio*	*Degradation (% as mannuronic acid)*
Short-chain poly-G	0.17	33
Alginate IV	0.40	36
Poly-MG	1.22	23
Long-chain poly-M	5.30	14
Fruiting bodies[a]	9.0	8
Short chain poly-M	15.8	0.7

[a]From *Ascophyllum nodosum.*

The enzyme acting on poly-G blocks had a K_m of 1.1×10^{-4}M. Initial action of the enzyme on poly-G blocks produced a series of oligosaccharides, but prolonged action gave three products with R_F values (t.l.c.; silica gel; butan-1-ol–acetic acid–water, 2:1:1) of 0.215 (*A*), 0.145 (*B*), and 0.09 (*C*), respectively. Separation of the products was achieved on Biogel P-4, the eluate being monitored by u.v. absorption (230 nm) and by testing for uronic acid[15]. The three products *A*, *B*, and *C* were each detected by both methods, suggesting that each contained the unsaturated moiety **4** as one component. Quantitative recovery was not possible, as each product required desalting after separation, but from the areas under the elution curves, the mol. proportions were estimated approximately to be *A*, 1.0; *B*, 1.0; and *C*, 0.1. Confirmation that each contained the unsaturated moiety **4** was obtained by periodate oxidation and formation of the chromophore with thiobarbituric acid, showing the typical absorption[13] at 550 nm. The d.p. of each oligosaccharide was estimated by comparison of its reducing power with that of guluronic acid, and the values were *A*, 2.3; *B*, 2.75; and *C*, 3.8, suggesting a di-, tri-, and tetra-saccharide, respectively. Following the procedure of Taylor and Conrad[16], the trisaccharide *B* was lactonised, and then reduced with sodium borohydride and hydrolysed with acid. Examination of the products by t.l.c., and by paper electrophoresis in borate buffer, revealed the presence of glucitol and an electrophoretically non-migrating substance. The reducing end-unit must have been L-guluronic acid (giving glucitol), and the internal unit must also be this acid (giving 1,6-anhydro-L-gulose). Control experiments with poly-M, and

with poly-MG blocks, showed that both mannose and mannitol were readily detected under these conditions, but that the L-guluronic acid units in the latter appeared as the non-migrating, anhydro derivative on electrophoresis. Assuming the nature and configuration of the linkages in alginic acid, the structures of *A*, *B*, and *C* were concluded to be Δ-G, Δ-G-G, and Δ-G-G-G, respectively, where Δ is the unsaturated moiety **4** ,and -G is a 4-linked α-L-guluronic acid unit.

CONCLUSION

The extracellular enzyme from *Klebsiella aerogenes* type 25 is an alginolyase with specificity for α-L-guluronosyl linkages in alginate; β-D-mannuronosyl linkages are not attacked. The end products of its action on poly-G blocks are mainly the unsaturated di- and tri-saccharides, with smaller amounts of the unsaturated tetrasaccharide, suggesting that the smallest unsaturated oligosaccharide that can be readily cleaved is the pentasaccharide, no trace of which was detected in the products. Investigation of the action pattern of this enzyme is continuing.

EXPERIMENTAL

General methods. — Protein was monitored by absorption at 280 nm, but for accurate determination, the method of Hartree[17] was used with bovine serum albumin as standard. Carbohydrate was detected with phenol–sulphuric acid[18]. Reducing powers of digests were measured with the dinitrosalicylate reagent[19], with D-mannuronic acid as standard; total uronic acids by the method of Blumenkrantz and Asboe-Hansen[15]; and unsaturated uronic acids by absorption at 230 nm[7] or by the periodate–thiobarbituric acid method[13]. Bacterial numbers in liquid cultures were monitored by absorption at 440 nm. Silica gel-G t.l.c. plates were developed with butan-1-ol–acetic acid–water (2:1:1) and sprayed with *p*-anisidine hydrochloride. Paper electrophoresis in borate buffer was performed as described previously[20].

Substrates. — Whole alginate from two commercial sources (samples IV and VII of Ref. 21) were kindly supplied by Dr. D. A. Rees. Long-chain poly-G blocks, and short-chain poly-M, poly-G, and poly-MG blocks were prepared from alginate as described previously[1,2]. Alginate having a high content of mannuronic acid was prepared from the intercellular substance of fruiting bodies of *Ascophyllum nodosum*[3].

Measurement of enzyme activity. — (*a*) *Viscometry.* The substrate solution contained alginate (0.66 g) and sodium chloride (1.75 g) in 100 ml of 0.05M phosphate buffer (pH 7.0). The substrate solution (5 ml) and enzyme solution (1 ml) were mixed at 25°, and 5 ml were rapidly transferred to an Ostwald viscometer maintained at 25°. The flow time was noted at intervals, and the flow times for water alone, and for substrate with water (1 ml) in place of enzyme, were also noted. Activity was calculated by the method of Hultin and Wanntrop[22].

(*b*) *Reducing power.* The substrate solution was identical to that above. The substrate solution (2 ml) and enzyme solution (1 ml) were incubated at 36° for 1 h,

and dinitrosalicylate reagent[19] (2 ml) was then added. The reducing power, as mannuronic acid, was determined as described previously[19]. One unit of activity is defined as the amount of enzyme that would liberate reducing power equivalent to 0.123 mg of mannuronic acid under the stated digest-conditions. The reducing power was directly proportional to the amount of enzyme added, up to 8 units of activity.

(*c*) *Increase in u.v. absorbance at 230 nm.* The above substrate solution (2 ml) and enzyme, in a total volume of 5 ml, were incubated at 36°. At intervals, the absorbance was measured in 1-cm cells, at 230 nm, against a blank of substrate solution (2 ml) diluted with water (5 ml). The increase in absorbance after 20 min was noted. One unit of enzyme, as defined above, produced an increase in absorbance of 0.178 in 20 min.

Isolation of enzyme. — The organism, *Klebsiella aerogenes* types 25, was obtained from the Public Health Laboratories, Colindale, London, on an agar slope. The growth medium contained Oxoid medium (13 g) and alginate (10 g) in water (1 litre), and was sterilised at 120° for 20 min. Medium (100 ml) was inoculated from the agar slope, and shaken at 36° for 27 h. Samples (1 ml) were transferred aseptically to 1-litre flasks containing medium (500 ml), which were then shaken at 36°. At intervals, bacterial growth was monitored; samples were withdrawn, and centrifuged to remove cells, and enzyme activity in the clear supernatant was assayed by viscometry (Fig. 1). Medium (3.5 l) in 500-ml batches was inoculated with a growing culture, shaken at 36° for 27 h, and then centrifuged to remove bacterial cells. Crude enzyme was precipitated at 4° by adding solid ammonium sulphate to 80% saturation. After 20 h at 4°, the enzyme was collected by centrifugation, redissolved in water (100 ml) at 0°, dialysed for 48 h against changes of 10mM phosphate buffer (pH 7), and freeze-dried (yield, 2.4 g).

DEAE Sephadex A-50 gel was equilibrated for 24 h with 0.15M imidazole buffer (pH 7.5), stirred for 24 h with 0.1M potassium chloride in 15mM imidazole buffer, and then packed in a column (2.5 × 50 cm). The column was eluted with 0.1M potassium chloride (2 vol.) at 4° before enzyme (400 mg) in water (2 ml) was loaded on the column, which was then eluted with 0.1M potassium chloride. Fractions (10 ml) were collected, and assayed for protein by u.v. absorption and for enzyme by the reducing-power method. Enzyme was eluted as a single peak (fractions 11–14), and protein as a broad peak (fractions 19–40). The active fractions were dialysed against 10mM phosphate buffer (pH 7) and freeze-dried (yield, 82 mg) (see Table I).

Properties of enzyme. — The pH optimum was determined by the reducing-power method using standard digests in phosphate buffer over the pH range 5–9. A broad optimum, centred at pH 7.0, was obtained. To study the effect of added salts, digests (2 ml) were prepared containing alginate (60 mg) in phosphate buffer (pH 7.0, 5mM) and the added salt. Enzyme (2 mg) in water (1 ml) was added, and the reducing power was determined after incubation for 1 h at 36° (Table II). To substrate solution (100 ml), as used for measurement of enzyme activity, enzyme (20 mg) in water (50 ml) was added. The relative viscosity was then determined on samples withdrawn at intervals, and the same samples, diluted with water (1 vol.), were used to determine

the absorbance at 230 nm (Fig. 2). Enzyme action on other polysaccharides was studied by incubating the polysaccharide (20 mg) in water (2 ml) with 0.1M acetate buffer (pH 6.0; 1 ml) and enzyme (10 mg) in water (1 ml) at 35° for 1 h. Blank digests contained water (1 ml) instead of enzyme. The reducing power was then determined with the dinitrosalicylate reagent. Only amylose and glycogen samples showed an increase in reducing power.

Action on alginate fractions. — Whole alginate or blocks (25 mg) and sodium chloride (0.25 g) in 0.05M phosphate buffer (25 ml) were incubated with enzyme (380 units) for 20 h at 36° under a layer of toluene. Further enzyme (300 units) was then added and incubation continued for 24 h. Portions (3 ml) of each digest were removed for determination of reducing power (Table III) and increase in absorbance at 230 nm. Digests incorporating substrate, but no enzyme, were used as controls, and their reducing power and absorbance at 230 nm were deducted from those of the enzyme digests. Long-chain poly-G blocks (~100% G) were used to determine the Michaelis constant. The substrate solution contained poly-G (12.2 mg) in 0.05M phosphate buffer (pH 7.0, 50 ml), *i.e.*, 0.99×10^{-6} mol of uronate per ml. The enzyme solution contained 16 mg in the same buffer. Digests containing substrate solution (0.2–0.4 ml), and buffer to 2 ml, were incubated with enzyme (1 ml) at 36° in 1-cm thermostatted cuvettes in a Unicam SP800 spectrophotometer, and the absorbances at 230 nm were plotted. From the graphs, the initial rates were determined, and a Lineweaver–Burke plot was used to calculate K_m.

Characterisation of products from poly-G. — Long-chain poly-G blocks (150 mg) and sodium chloride (0.5 g) in 0.05M phosphate buffer (pH 7; 75 ml) were incubated at 36° with enzyme (900 units) under a layer of toluene. Portions (0.2 ml) were withdrawn at intervals, and diluted with water (5 ml) for measurement of absorbance at 230 nm. After 80 h, the absorbance remained constant, and the digest was then freeze-dried (yield, 810 mg). The products were desalted on a column (185 × 2 cm) of Sephadex G-10 (previously swollen in distilled water), and eluted with water, fractions being tested for oligosaccharides (absorption at 230 nm) and salt (silver nitrate). The oligosaccharide fractions were freeze-dried, and examined by t.l.c. (see Discussion). Biogel P-4 (100–200 mesh) was swollen in 0.1M sodium sulphate and packed into a column (190 × 2 cm). The oligosaccharides in 0.1M sodium sulphate (2 ml) were loaded on the column and eluted with the same solution; fractions (2 ml) were collected, and assayed by absorbance at 230 nm. Selected fractions were also tested for uronic acid by the method of Blumenkrantz and Asboe-Hansen[15]. Three peaks, each detected by both methods, were eluted: *C* (fractions 130–151), *B* (fractions 152–172), and *A* (fractions 174–194). Oligosaccharides *B* and *C* were desalted on Sephadex G-10 as described above; *A* could not be desalted completely by this method but was desalted on a column of charcoal–Celite[23].

Each oligosaccharide (~1 mg) was treated with periodate, and the products were then treated with thiobarbituric acid[13]. The spectra of the chromogens were recorded over the range 450–700 nm on a Unicam SP800 spectrophotometer. Each spectra showed λ_{max} 550 nm, with a shoulder at 510–515 nm. The reducing power of

each oligosaccharide was compared with that of authentic L-gulose by using the dinitrosalicylate reagent (see Discussion).

Oligosaccharide *B* (20 mg) was lactonised, and then reduced with sodium borohydride on the pH stat by the method of Taylor and Conrad[16]. The reduced product was hydrolysed with M-sulphuric acid (50 ml) at 100° for 1 h, neutralised (barium hydroxide), and concentrated. The product was deionised on a mixed-bed resin [BioDeminrolit (carbonate form)], and then examined by t.l.c., and by paper electrophoresis in borate buffer. Glucitol and an electrophoretically non-migrating material were detected with the periodate–permanganate spray reagent[25]. Similar treatment of authentic L-gulose gave glucitol only, whereas poly-M and poly-MG blocks gave, respectively, products having the mobilities of mannose, and mannose plus the non-migrating material.

ACKNOWLEDGMENTS

We thank Mr. T. Pitt for the culture of the bacterium, Dr. D. A. Rees for the determination of M/G ratios of alginate fractions, and the S.R.C. and Unilever Research Ltd. for financial support.

REFERENCES

1 A. HAUG, B. LARSEN, AND O. SMIDSRØD, *Acta Chem. Scand.*, 21 (1967) 691–704.
2 A. HAUG, B. LARSEN, AND O. SMIDSRØD, *Acta Chem. Scand.*, 20 (1966) 183–190.
3 A. HAUG, B. LARSEN, AND O. SMIDSRØD, *Carbohydr. Res.*, 32 (1974) 217–225.
4 R. KOHN, I. FURDA, A. HAUG, AND O. SMIDSRØD, *Acta Chem. Scand.*, 22 (1968) 3098–3102.
5 G. T. GRANT, E. R. MORRIS, D. A. REES, P. J. C. SMITH, AND D. THOM, *FEBS Lett.*, 32 (1973) 195–198.
6 Y. KASHIWABARA, H. SUZUKI, AND K. NISIZAWA, *J. Biochem. (Tokyo)*, 66 (1969) 503–512.
7 J. PREISS AND G. ASHWELL, *J. Biol. Chem.*, 237 (1962) 309–316.
8 J. MADGWICK, A. HAUG, AND B. LARSEN, *Acta Chem. Scand.*, 27 (1973) 711–712.
9 Y. SHIRAIWA, K. ABE, S. F. SASAKI, T. IKAWA, AND K. NISIZAWA, *Bot. Mar.*, 18 (1975) 97–104.
10 K. NISIZAWA, S. FUJIBAYASHI, AND Y. KASHIWABARA, *J. Biochem. (Tokyo)*, 64 (1968) 25–37.
11 L. A. ELYAKOVA AND V. V. FAVOROV, *Biochim. Biophys. Acta*, 358 (1974) 341–354.
12 A. LINKER AND R. S. JONES, *J. Biol. Chem.*, 241 (1966) 3845–3851.
13 A. WEISSBACH AND J. HURWITZ, *J. Biol. Chem.*, 234 (1959) 705–709.
14 E. HULTIN, *Acta Chem. Scand.*, 3 (1949) 625–629.
15 N. BLUMENKRANTZ AND G. ASBOE-HANSEN, *Anal. Biochem.*, 54 (1973) 484–489.
16 R. L. TAYLOR AND H. E. CONRAD, *Biochemistry*, 11 (1972) 1383–1388.
17 E. F. HARTREE, *Anal. Biochem.*, 48 (1972) 422–427.
18 M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH, *Anal. Chem.*, 28 (1956) 350–356.
19 G. NOELTING AND P. BERNFELD, *Helv. Chim. Acta*, 31 (1948) 286–290.
20 D. M. BOWKER AND J. R. TURVEY, *J. Chem. Soc., C*, (1968) 983–988.
31 A. PENMAN AND G. R. SANDERSON, *Carbohydr. Res.*, 25 (1972) 273–282.
22 E. HULTIN AND I. WANNTROP, *Acta Chem. Scand.*, 20 (1966) 2667–2677.
23 J. F. BATEY AND J. R. TURVEY, *Carbohydr. Res.*, 43 (1975) 133–143.
24 R. U. LEMIEUX AND H. F. BAUER, *Anal. Chem.*, 26 (1954) 920–921.