Carbohydrate Polymers **19** (1992) 51–59

Enzymatic microassay for the determination and characterization of alginates

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(Received 10 December 1991; accepted 13 January 1992)

When alginate is split by an alginate lyase, an unsaturated unit with a strong absorbance at 230 nm is formed at the new non-reducing end. At excessive enzyme levels, the absorbance will approach an apparent endpoint level that reflects the initial substrate concentration. On this basis, a simple and rapid assay has been developed to detect and quantify alginates in solution in the 0·01–1 mg/ml concentration range. A combination of purified guluronate lyase from *Klebsiella pneumoniae* and purified mannuronate lyase from *Haliotis tuberculata* is applied to eliminate the influence of the specific composition of the alginates. Applied separately and in combination, these enzymes may also give information about the alginate structure in accordance with their block distribution structure determined by NMR.

INTRODUCTION

Alginates are salts of alginic acid, a linear copolymer of α 1-4 L-guluronic acid and β 1-4 D-mannuronic acid. The uronic acid residues are organized in blocks of polymannuronic acid (MM), polyguluronic acid (GG), and heteropolymeric sequences containing alternating structures (MG) (Haug *et al.*, 1974). The actual chemical composition and structure of alginates depend strongly on their origin; in brown algae they depend not only on the species, but also on type and age of the tissue, season of the year and on growth conditions.

Many investigators have reported on the determination of alginate and/or uronic acids obtained by hydrolysis of alginic acid (Jensen & Sunde, 1955). Uronic acids may be quantified by the classical phenol-sulfuric acid method (Dubois *et al.*, 1956). Jensen *et al.* (1985) estimated the alginate content in algae by transferring the material to alginic acid, then to Ca-alginate, and finally measuring Ca by atomic absorption. Murata *et al.* (1991) applied a selective colorimetric reagent for carboxylic acid for determination of alginates and their hydrolysates. Dye-based assays were also evaluated by Ragan (1990), but not recommended.

These methods have different characteristics, inherent limitations and sources of error, generally related to inaccuracies and losses introduced during extraction, separation and treatment of the material, or related to calibration procedures biased by variations in the uronic acid composition. Most importantly, none of the methods available can be considered as rapid and simple. A screening test would be particularly helpful when monitoring a large number of samples. This is always the case when biological variability must be taken into consideration. Typical examples in research occur when analyzing the presence or biosynthesis of alginate in bacteria or algae. In the alginate industry, it is of course essential to monitor batch variations in raw materials and products.

Alginate lyases catalyze cleavage of the alginate chain by a β-elimination reaction and create an unsaturated uronic acid residue at the new non-reducing end. This unit will absorb radiation strongly in the UV region at 230–240 nm, and alginate lyase activity may therefore be easily detected as an increased absorbance at these wavelengths (Nakada & Sweeny, 1967). The final end product is apparently a mixture of small oligomers with low DP (Boyd & Turvey, 1978; Currie, 1983).

Alginate lyases are found in a variety of organisms, including marine molluscs, echinoderms, bacteria and fungi (Boyen *et al.*, 1990*a*, 1990*b*; Butler *et al.*, 1990; Gacesa & Wusteman, 1990). It has been shown that different lyases can have preferences for different types of alginate blocks or uronic acids. However, as pointed

Carbohydrate Polymers 0144-8617/92/$05.00

out by Haugen *et al.* (1990), the term specificity should be used with caution for enzymes acting on copolymers with non-repeating structures. The author has chosen to classify alginate lyases roughly as mannuronate (M-) lyases, defined by an incapability to cleave polyguluronic blocks, or guluronate (G-) lyases, when inactive to a polymannuronic substrate. In this sense, lyases from marine molluscs generally appear to be endo-enzymes of the mannuronate type (Boyen *et al.*, 1990*a*). This has been particularly well documented for the abalone (*Haliotis* sp.) alginate lyase by Nakada and Sweeny (1967), Boyen *et al.* (1990*a*), Haugen *et al.* (1990) and others. Guluronate lyases have been found in some bacteria (Brown & Preston 1991), including *Pseudomonas alginovora* (Boyen *et al.* (1990*b*) and *Klebsiella pneumoniae* (Boyd & Turvey, 1977; Haugen *et al.*, 1990; Aasen *et al.*, 1992).

Alginate lyases are examples of polymer-cleaving enzymes, where fundamental aspects such as proper definitions of substrate and product concentrations in the Michaelis–Menten sense may make kinetic analysis difficult. At excessive enzyme levels, the absorbance will approach or increase to an apparent endpoint level that depends on the initial substrate concentration. A simple assay for substrate detection should preferably work at conditions where product formation has reached a stable level.

The present investigation was undertaken to evaluate the use of M- and G-specific alginate lyases as tools in a screening test to quantify and characterize alginates, by applying the increase in UV absorbance recorded at the apparent endpoint in order to estimate the initial substrate concentrations.

MATERIALS AND METHODS

Alginates

The composition of the different alginates applied as substrates in the following studies are summarized in Table 1. Alginates from *Laminaria digitata* were supplied by Protan A/S (Drammen, Norway); those from *Macrocystis pyrifera* by Sigma (St. Louis, USA). The uronic acid block composition was characterized by nearest-neighbour frequencies determined by NMR spectroscopy (Grasdalen, 1983). Reference samples used in quantitative comparisons were purified by repeated dialysis, ethanol precipitation, ethanol and ether washings and drying.

Alginate fragments of extreme block composition were prepared as described by Haug *et al.* (1966; 1974). Material dominated by high content of guluronic acid (GG) was prepared from *Stilophora rhizoides*. Samples with high content of mannuronic acid were obtained from *Sargassum* sp., while fragments with high transition frequencies (MG) came from *Ascophyllum nodosum*. All fragments had an average $DP_n > 20$. Table 2 summarizes the NMR data for the block distribution of these samples.

Enzymes

Alginate M-lyase was prepared from fresh *Haliotis tuberculata*, collected at Roscoff, Brittany. Preparative purification was performed by ion-exchange chromatography as described by Boyen *et al.* (1990*a*).

Alginate G-lyase was obtained from *Klebsiella pneumoniae* cultivated in fermenters up to a volume of 150 liters as described by Aasen *et al.* (1992). Large-scale purification was based on hydrophobic interaction chromatography (Aasen *et al.*, 1992).

After purification, both enzymes proved to be stable when stored as frozen stock solutions.

Assays

Alginate lyase *activities* were determined at 20°C in 50 mM Tris-HCl buffered to pH 7·5, as described by Haugen *et al.* (1990). Volumes of 2 ml buffer, 0·5 ml substrate solution and 0·5 ml enzyme were mixed directly in a 10 mm quartz cuvette, to give concentrations of 1% NaCl and 0·1% alginate substrate. Standard substrate was alginate from *L. digitata*, containing at least 25% of MM, MG and GG blocks. The increase in absorbance at 230 nm was recorded continuously in a Shimadzu UV-260 Spectrophotometer to verify linearity, and the enzyme diluted and

Table 1. Composition of alginates applied in tests (data obtained by NMR)

Sample: source	F_M	F_G	F_{MM}	F_{MG}	F_{GM}	F_{GG}
1: *L. hyperborea* outer cortex	0·25	0·75	0·16	0·09	0·09	0·66
2: *L. hyperborea* LF10/60	0·32	0·68	0·20	0·12	0·12	0·56
3: *L. digitata*	0·59	0·41	0·43	0·16	0·16	0·25
4: *M. pyrifera* Kelco 55066A	0·57	0·43	0·35	0·22	0·22	0·21
5: *D. antarctica*	0·66	0·34	0·45	0·21	0·21	0·13
6: *A. nodosum*FMI	0·82	0·18	0·78	0·05	0·05	0·13
7: *L. digitata*	0·57	0·43	0·43	0·14	0·14	0·29

Table 2. Composition of alginate block fragments (data obtained by NMR)

Sample: source	F_M	F_G	F_{MM}	F_{MG}	F_{GM}	F_{GG}
1: 'MG' type	0·63	0·37	0·34	0·29	0·29	0·08
2: 'MM' type	0·92	0·08	0·88	0·04	0·04	0·04
3: 'GG' type	0·05	0·95	0·0	0·05	0·05	0·90

recordings repeated if necessary. Enzyme activity is reported in units (UA) defined as the increase in absorbance units per minute.

Enzymatic characterization of *substrates* was performed in the same system. The pH of the sample solutions to be tested was adjusted if outside the interval pH 7–8. Volumes of 4 ml buffer and 2 ml alginate test solution, or 2 ml and 1 ml, respectively, were mixed and the background absorbance recorded. A small volume, 100 μl unless otherwise stated, of 1 UA/ml stock solution of purified alginate M- and/or G-lyase was added to all samples. The enzyme background was recorded separately in a blank sample without alginate. Absorbance recordings, unless otherwise stated, were repeated until a stable level was reached.

Application studies

Alginate solubility was studied by adding 40 ml of water containing various amounts of $CaCl_2$ to 40 mg of commercial alginate from *L. digitata* in tubes continuously stirred overnight. After centrifugation at 15 000 *g* for 1 h, the alginate content of the supernatant was determined as described above. Uronic acids were also determined independently by the phenol-sulfuric acid method (Dubois *et al.*, 1956).

Alginates were extracted from *Laminaria digitata* tissue by a procedure modified from Haug (1964). Pieces of tissue were chopped, dried, ground in a mortar and weighed in aliquots of 5–50 mg powder per sample. Acid treatment was performed by adding 10 ml of 0·2 N HCl and incubating overnight with continuous stirring. Solid material was removed by centrifugation and washed twice with 0·2 N HCl. The pellet was resuspended in 10 ml 0·1 N $NaHCO_3$, incubated for 2 h, pH adjusted to 7·6–7·9 with $NaHCO_3$/HCl and incubated overnight. The next day, the pH was recorded and adjusted to pH 7·0, the volume recorded, the sample centrifuged and the alginate content of the supernatant determined as described above.

RESULTS AND DISCUSSION

Linearity

Analysis of time progress curves revealed that stable endpoint levels could be obtained, within 2 h for 0·01–0·2 UA of the *Haliotis* mannuronate lyase acting on a 0·1% substrate of alginate from *Laminaria digitata* (results not included). This apparent endpoint was, however, dependent on the enzyme concentration, reflecting a product inhibition effect that will be discussed in detail elsewhere (Østgaard & Larsen, unpublished results). The enzyme dose must therefore be standardized within certain conditions in order to obtain a constant degree of conversion, that is, a range where product formation is directly proportional to the initial substrate concentration. The G-lyase of *Klebsiella pneumoniae* has not been studied in such detail, but data suggest a behavior similar to that of M-lyase (results not included).

On this basis, 0·1 UA of both enzymes and 3 h of incubation were selected as standard conditions for the author's assay. Calibration curves obtained in this way are presented in Fig. 1. Linearity was clearly verified.

It is important to note that substrate concentrations

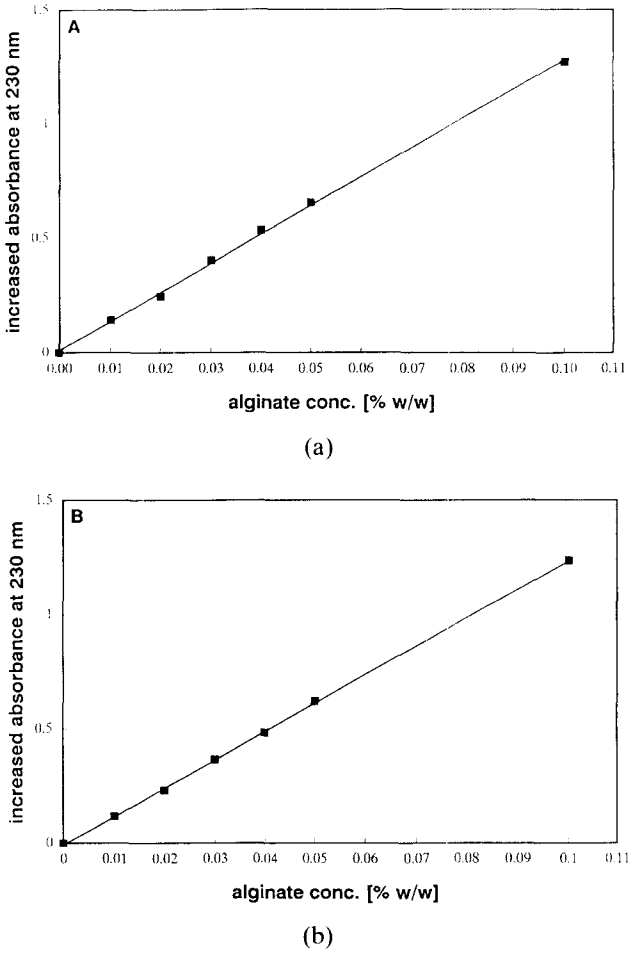


Fig. 1. Product formation, recorded as increased absorbance at apparent endpoint level (3 h), shown as a function of initial substrate concentration. The alginate substrate was of *L. digitata* type, sample 7 in Table 1. Curves were obtained by linear regression. Enzyme added: (a) 0·1 UA of M-lyase from *Haliotis tuberculata*; (b) 0·1 UA of G-lyase from *Klebsiella pneumoniae*.

exceeding a certain upper limit will be erroneously underestimated. According to Fig. 1 standard test conditions should be restricted to substrate concentrations corresponding to 0·1% of *L. digitata* alginate, or roughly an increase of 1 absorbance unit for both enzymes in the author's instrument. Samples exceeding this level should be diluted and retested.

Sensitivity

The lower detection limit of the standard assay will depend on many factors. Figure 2(A) shows a calibration curve for M-lyase at low concentrations of *L. digitata* alginate. The lowest concentration, 0·001%, corresponds to 10 μg of alginate in a test volume of 1 ml. Apparently, alginate may easily be quantified by this method even at such low concentrations. Figure 2(B) shows a corresponding curve for the G-lyase. The linear regression curve did not go through the origin in this case, reflecting an error or instability in the recorded enzyme background absorbance. Both enzymes are partially purified, giving a much lower specific activity (higher protein content) for the G-lyase. Thus, the standard 0·1 UA gave a background absorbance of 0·005 for the M-lyase and 0·26 for the G-lyase, which is more than a factor of 10 times higher than the increase in absorbance recorded at the lowest test concentration.

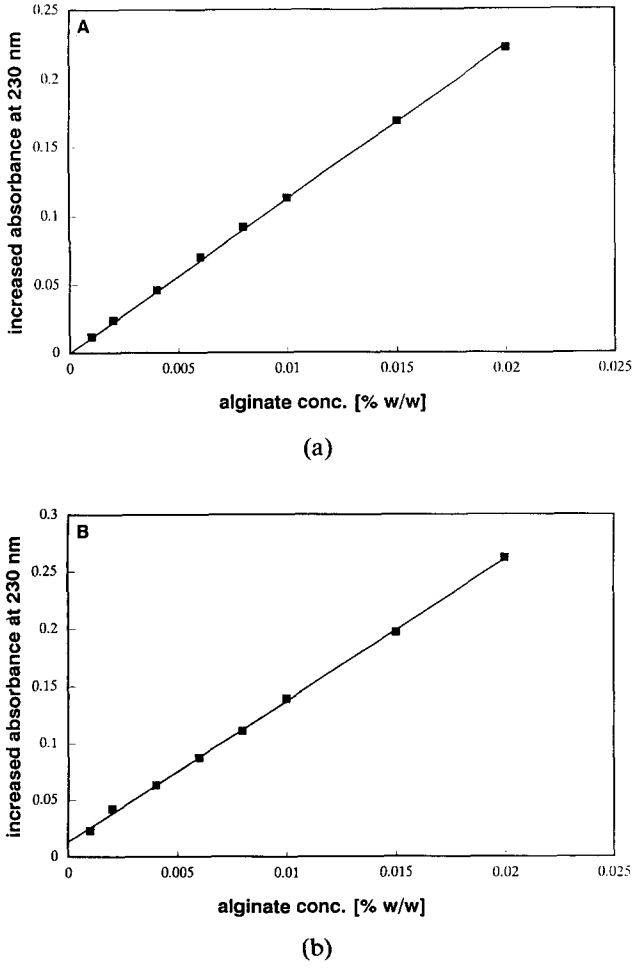


Fig. 2. Product formation, recorded as increased absorbance at apparent endpoint level (3 h), shown as a function of initial substrate concentration. The alginate substrate was of *L. digitata* type, sample 7 in Table 1. Curves were obtained by linear regression. Enzyme added: (a) 0·1 UA of M-lyase from *Haliotis tuberculata*; (b) 0·1 UA of G-lyase from *Klebsiella pneumoniae*.

It is evident that when working with alginate in the μg scale, the main problem will be the accuracy and stability of the background absorbance introduced by the enzyme and the impurities of the test sample. Even when such biased errors are avoided, the practical detectability will depend strongly on the sensitivity and stability of the spectrophotometer in use. Sensitivity may of course be improved by increasing the optical path length and/or the number of replicates for each sample. This will, however, also increase the sample size needed. Subtle balancing at a statistical level of significance is not recommended for a screening type of test.

Sample purity

The purity of the sample may also affect the recordings. Proteins absorb strongly even at 230 nm, as illustrated by the enzyme preparations above. When solutions of bovine serum albumin (BSA) alone were tested at the standard conditions, a linear increase in absorbance was observed for concentrations up to 0·2% BSA, corresponding to an absorbance value of 2·6. When increasing amounts of BSA were added to a test sample of 0·04% of *L. digitata* alginate assayed by M-lyase and G-lyase, variability was moderate, within ±8·5% (SD) for M-lyase and ± 2·5% (SD) for the G-lyase data (see also Fig. 3(A)). This is acceptable for most screening purposes. Furthermore, samples may always be diluted to levels within the absorbance range of the instrument. Roughly, the background absorbance of BSA was at the same level as the increase in absorbance of the enzyme assay for a similar alginate concentration (weight/weight). Therefore, in a solution containing a 100 times more protein than alginate, the expected enzymatic increase in absorbance would be at 1% of the background level, and hardly detectable. If protein contamination constitutes 90% of the dissolved material or less, the assay should work well, within the limits given above.

Samples at extreme pH exceeding the buffer capacity of the test buffer will affect enzyme activities, although not necessarily the final endpoint levels. It should also be noted that these enzymes have quite wide pH optima. Nevertheless, sample pH should be checked and adjusted if necessary. Enzyme activities also depend on salt concentration. The G-lyase of *Klebsiella pneumoniae* differs from that of *Pseudomonas alginovora*

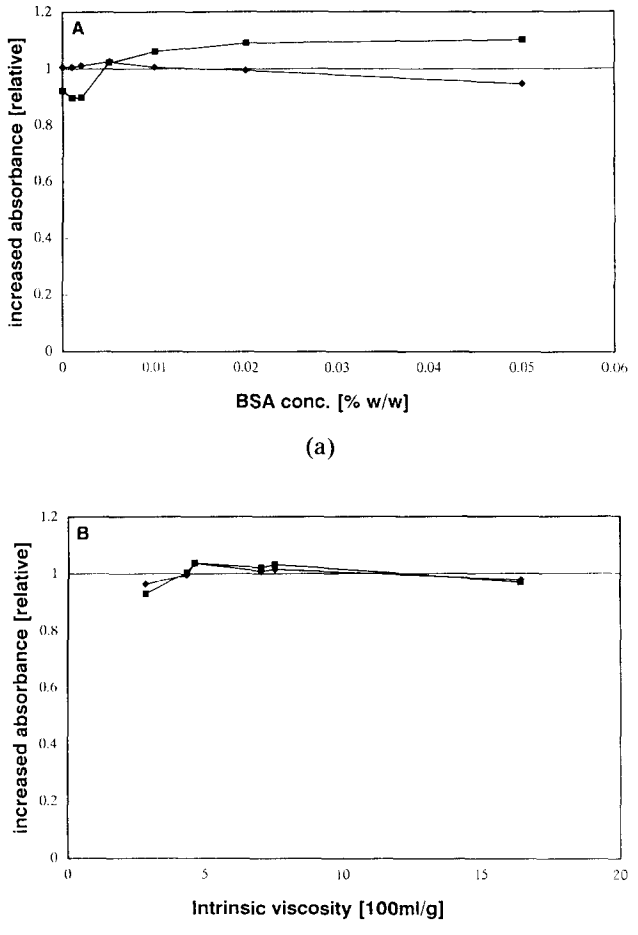


Fig. 3. Product formation, recorded as increased absorbance at apparent endpoint level (3 h) relative to the mean value, obtained with an alginate substrate of *L. digitata* type, sample 7 in Table 1. Enzyme added was 0·1 UA of M-lyase from *Haliotis tuberculata* (■) or 1 UA of G-lyase from *Klebsiella pneumoniae* (♦). Data shown as a function of: (a) added concentrations of bovine serum albumin; (b) molecular weight of alginate substrate, characterized by its intrinsic viscosity.

by its particular calcium dependence (results not included). Addition of small amounts of Ca^{2+} may compensate for the reduced enzymatic activity otherwise observed at high salt concentrations. The calcium content may also affect the final degree of conversion. When solutions (0·025%) of the different alginate substrates listed in Tables 1 and 2 were tested after addition of 1 mM $CaCl_2$ to the standard buffer, the mean absorbance at 0·5–4·5 h increased by 11–20%. The M-lyase was apparently unaffected by additional calcium (results not included). The calcium dependence of the *Klebsiella* enzyme should be taken into account in order to minimize errors in comparative studies of different alginate samples with unknown calcium contamination. For rough and rapid screening purposes, a deviation of maximum 20% may be acceptable. If not, the calcium content should be standardized.

Substrate characteristics

The molecular weight of the alginate substrate may be of significance to the enzyme affinities and the final total product formation recorded. One sample of *L. hyperborea* outer cortex alginate was broken down in steps by sonication to a series of samples with intrinsic viscosities from 16·4 to 2·8 (100 ml/g). All samples were tested at 0·025% in the standard assay, and the results for both M- and G-lyase are shown in Fig. 3(B). The variability was within ±4·2% (SD) for M-lyase and ± 2·7% (SD) for G-lyase, which is negligible for any screening purpose. These small variations appeared to be non-random and very similar for both enzymes, possibly due to slight variations in the humidity of the non-dried samples. It is evident that molecular size must have some effect for very small substrate molecules, particularly since the final product appeared to be at the oligomer level for both enzymes (Haugen *et al.*, 1990). The smallest molecules tested, the block fragments of Table 2, had an average DP_n far lower than the smallest alginates of Fig. 3, roughly estimated to an average DP_n of 70 according to the Mark–Houwink equation (Smidsrød & Haug, 1968) (see also below).

The chemical composition and block structure of the substrate must be regarded as essential for the outcome of tests using different lyases with different specificities. This may be revealed by using the extreme block fragments given in Table 2. The samples were dried and tested at 0·04% in the standard assay of M-lyase and G-lyase. In the case of M-lyase acting on the alternating MG substrate, absorbance increased steadily from 89% at 2 h to 105% at 4 h, relative to the 3 h level. All other recordings of significance were stable within ±4% for incubation periods of 2–4 h. Within these limits, 3 h values are considered generally acceptable as estimates of the final endpoint value for screening purposes. These values are summarized in Table 3. The data of Tables 2 and 3 verify that the specificity of both enzymes can be classified as an inability to catalyze the cleavage of homogenous polyuronic blocks of opposite type, while both effectively facilitated the breakdown of the substrate enriched in alternating MG sequences.

The enzymes cannot be expected to have identical affinities or abilities of substrate utilization. A 'best fit' to the complete data set obtained at 3 h (principally,

Table 3. Product formation (recorded as increased absorbance at 230 nm) after 3 h of incubation with 0·1 UA alginate lyase and 0·04% of substrates specified in Table 2

Sample: source	M-lyase	G-lyase	Both
1: 'MG' type	0·451	0·567	0·762
2: 'MM' type	0·634	0·092	0·694
3: 'GG' type	0·048	0·812	0·794

Table 4. Models and estimates of alginate lyase specificities, in the sense of relative degrees of substrate conversion, to different types of alginate blocks

Method/substrate	M-lyase specificity to blocks of:			G-lyase specificity to blocks of:		
	MM	MG	GG	MM	MG	GG
Model (see text)/alginate fragments (Table 2)	1	0·67	0	0	1·32	1·20
Regression (see text)/alginates (Table 1)	1	0·69	0	0	1·16	1

nine equations and four unknowns) is presented in Table 4. According to this model, M-lyase will prefer a poly-M substrate, while the G-lyase will be most effective on the alternating MG substrate. As shown in Fig. 4, this flexible model gave, as expected, a good fit to experimental data.

It should be noted that even in the presence of both enzymes, the values recorded at 3 h correspond to 68–77% of those of intact alginates presented below. This difference may be related to the very low molecular weight of the block type substrates.

Total estimates

So far, absolute alginate concentrations can only be obtained when a pure reference sample with the same chemical composition is available for calibration. As illustrated above, the problems of chemical differences between test samples may be minimized by including both M- and G-lyase in the test. It is evidently a considerable overlap in the substrate specificities of the two enzymes. They should therefore be applied simultaneously.

Figure 5(A) shows the results of testing all the alginates of Table 1 after purification and dehydration. The test concentration was 0·04%. The relative variability was within ±4·1% (SD), and the extreme values were only 4·5% above and 5·3% below the mean value. Considering the wide differences in chemical composition, this accuracy is expected to be generally valid for algal alginates. It is concluded that alginates of widely different or unknown composition can be quantified in

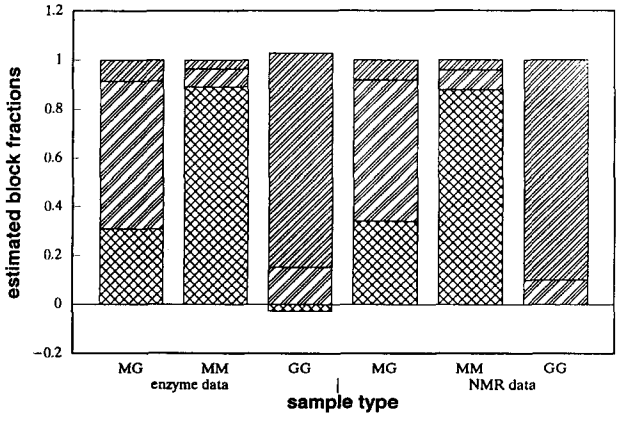
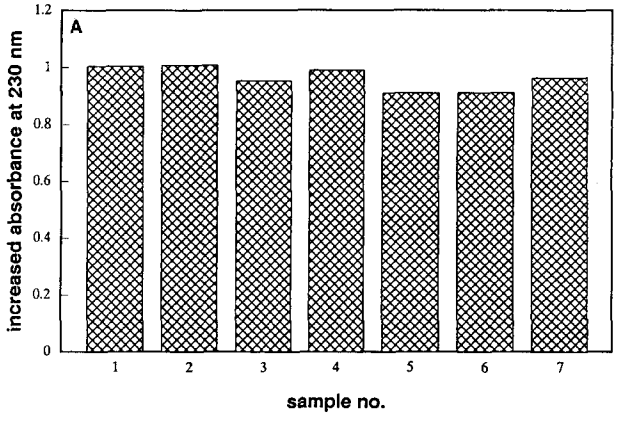
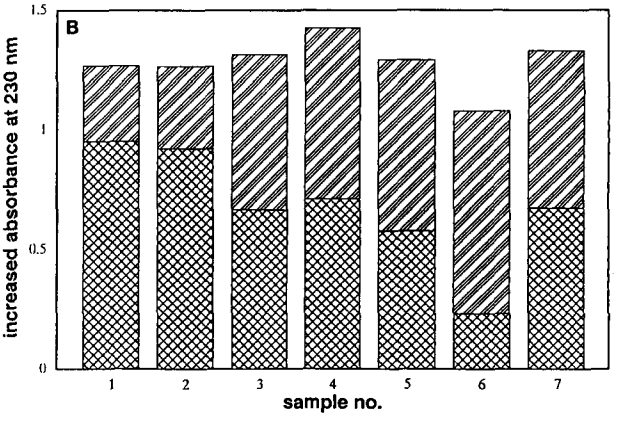


Fig. 4. Alginate block composition of three different alginate fragments enriched in different block types (Table 2), shown as stacked bars; from bottom to top: MM fraction, MG fraction and GG fraction. Bars to the left show results obtained by the model of Table 4 based on enzyme data; bars to the right show results obtained by NMR.



(a)



(b)

Fig. 5. Product formation, recorded as increased absorbance at apparent endpoint level (3 h), shown for an initial concentration of 0·04% of alginate substrates identified by sample numbers given in Table 1. Enzymes added: (a) 0·1 UA of M-lyase from *Haliotis tuberculata* and 0·1 UA of G-lyase from *Klebsiella pneumoniae* added simultaneously; (b) data obtained with 0·1 UA of M-lyase from *Haliotis tuberculata* only stacked on top of data obtained with 0·1 UA of G-lyase from *Klebsiella pneumoniae* only.

this way within an expected calibration error of ±5%. This is considered as very good for most applications.

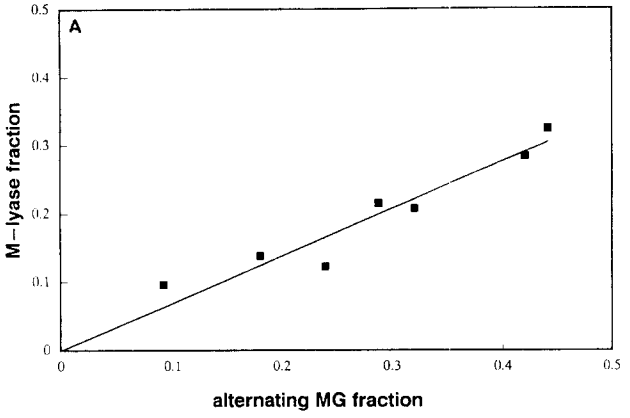
The relative degrees of conversion obtained for the block fragments in Table 4 may indicate that the MM blocks broken down by the M-lyase will contribute somewhat less than those split by the G-lyase when both enzymes are applied in combination. The samples with the highest MM content — samples 3, 5 and 6 (Table 1) — in fact gave the lowest values in Fig. 5(A), as expected. The differences between samples in Fig. 5(A) should therefore not be ascribed to an experimental random error.

Alginate characterization

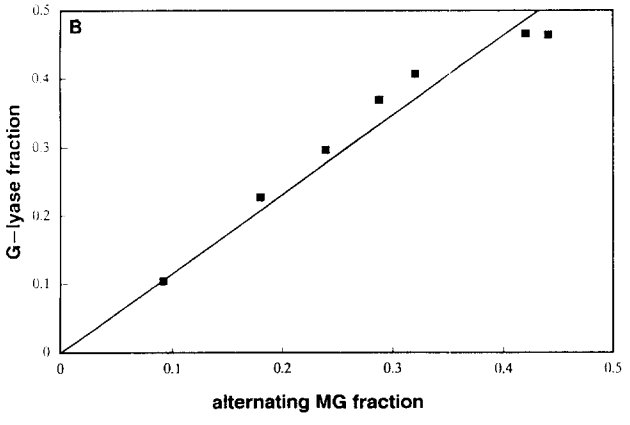
Figure 5(B) presents data obtained when the same samples were analyzed with M- and G-lyase in separate tests. The differences in sample composition and in substrate specificity are clearly illustrated. These enzymatic data may be related to data determined by NMR (Table 1) in many different ways. The overlap in substrate specificity of the two enzymes should be related to the alginate blocks with high transition (MG, GM) frequency, the alternating MG fraction. This overlap is reflected in the increase in the sum of the M- and G-lyase results compared to that of both enzymes combined (Fig. 5). A correlation was clearly observed when this increase was plotted as a function of the alternating fraction determined by NMR. As a first-order approximation, one may also assume that the enzymatically determined fractions will exceed the content of their respective homopolymeric blocks by a value proportional to the alternating fractions. If the enzymes have somewhat different abilities in breaking down the alternating structure compared to their respective homopolymeric blocks, the proportionality factor will be somewhat different from 1.

Figure 6 shows the results of using this model as a basis for linear regression, giving the coefficients listed in Table 4 as the best fit. These values are close to those derived independently for the block substrates, that is, 0·67 and 1·32/1·20 = 1·10, respectively (Table 4). It should be noted that these proportionality factors have been derived on a purely empirical basis, and that the exact values may change if experimental conditions such as the Ca^{2+} level are changed. Moreover, data have been recorded at an apparent experimental endpoint, and therefore do not necessarily reflect kinetic differences between block types (see also Haugen *et al.*, 1991). The equations given above do indicate that while the M-lyase of *Haliotis* is apparently splitting the homopolymeric blocks to a larger extent than the alternating structures, there is no such clear preference shown by the G-lyase of *Klebsiella*. It is not unlikely that this is related to differences in enzyme–substrate affinities for the different block types.

In conclusion, empirical constants such as those



(a)



(b)

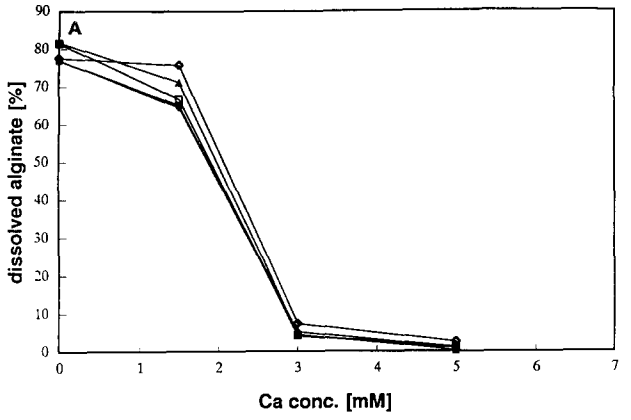
Fig. 6. Correlation between fractions determined by enzymatic degree of conversion and substrate content of MG blocks determined by NMR. Ordinate values show the ratio between data obtained by single enzyme exposure (Fig. 7(B)) and both enzymes combined (Fig. 7(A)), after subtraction of their respective homopolymeric blocks according to NMR (see also text). Single enzyme data were obtained with: (a) 0·1 UA of M-lyase from *Haliotis tuberculata*; (b) 0·1 UA of G-lyase from *Klebsiella pneumoniae*.

given in Table 4 may be used to obtain an enzymatically based estimate or rough characterization of the block composition of an unknown alginate. It is of course not recommended when NMR analysis is available.

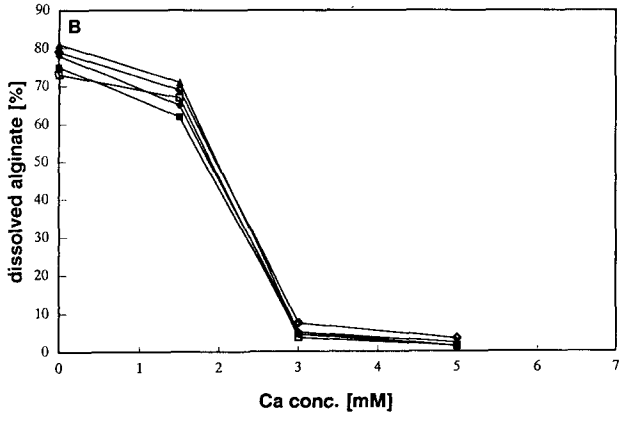
Applications

Alginate solubility

As a simple example of an application where the problems of alginate structure are avoided, Fig. 7(A) shows the solubility of a sodium alginate obtained from *L. digitata* at different calcium concentrations. Data obtained by the phenol-sulfuric method are shown in Fig. 7(B). Comparing both methods, 20 independent samples gave a correlation coefficient of 0·9978. The curves do not start at 100% because of the water content of the material. Although not emphasized



(a)



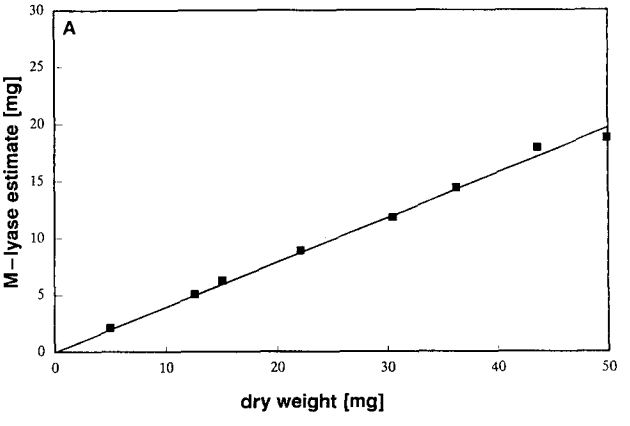
(b)

Fig. 7. Alginate solubility. The dissolved fraction of a 0·1% suspension of a commercial alginate of *L. digitata* type is shown as a function of the added $CaCl_2$ concentration. Data are given for five independent replicates. Results were obtained by: (a) enzymatic detection; (b) the phenol-sulfuric method.

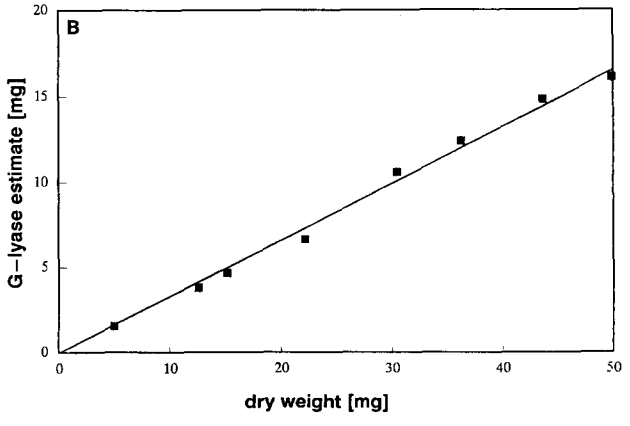
in the diagrams of Fig. 7, it should be noted that both methods gave the same consistent differences between curves also at high calcium levels. Since the enzyme test is run with $\frac{1}{3}$ of the sample volume, calcium levels during testing were up to 1·7 mM. This had apparently no significant effect on the measurements. Figure 7 shows a dramatic drop in solubility at 3 mM Ca^{2+}. This limit corresponds closely to the concentration generally needed to induce formation of a Ca-alginate gel. It is of fundamental importance for alginate extraction, solubility and gel formation during entrapment of living cells, as well as dissociation of the alginate of the cell wall when making protoplasts of brown seaweeds (Butler *et al.*, 1989).

Alginate content and composition in Laminaria digitata *tissue*

Freshly collected tissue gave a dry weight of 15·6% of the fresh weight. Figure 8 shows the results obtained by applying M- and G-lyase separately to extracts of different amounts of the dried material. Linearity was observed for both enzymes. The remaining pellet was subject to a second extraction. Although values were much lower and less accurate, linearity was observed also in this case (results not included). Most importantly, the second extraction gave values equivalent to 7% of the first extraction, corresponding to the relative volume of the pellet transferred. One neutral extraction is therefore considered as sufficient to obtain reliable results. Based on the results of Fig. 8, the alginate content was estimated to be 36·6% of the dry weight, with a guluronic content of 41%. This is in accordance with the typical values generally found for *L. digitata* blade by other methods (Table 1; see also Haug, 1964). It is concluded that the microextraction procedure and enzyme analysis will give reliable results when performed as described. The same methods are now being applied to analyze the alginate content of cells and protoplasts.



(a)



(b)

Fig. 8. Alginate content of *L. digitata* tissue. Enzymatic estimates of alginate content (calibrated with an alginate standard of slightly different composition) are shown as a function of dry weight of tissue applied for extraction. Data were obtained by: (a) 0·1 UA of M-lyase from *Haliotis tuberculata*; (b) 0·1 UA of G-lyase from *Klebsiella pneumoniae*.

ACKNOWLEDGMENTS

The skillful experimental assistance of Kurt Draget, and Ann-Sissel Ulset is gratefully appreciated. Kari Røbech and Wenche Strand kindly supplied alginate samples and NMR data. The author also wishes to thank Prof. Arne Jensen and Prof. Bjørn Larsen for helpful discussions and critical revision of the manuscript. This work was supported by the Bioenergy Research Programme of the Royal Norwegian Agricultural Research Council (NLVF).

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