Carbohydrate Polymers 110 (2014) 100–106

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Lyase-catalyzed degradation of alginate in the gelled state: Effect of gelling ions and lyase specificity

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ARTICLE INFO

Article history:
Received 19 December 2013
Received in revised form 19 March 2014
Accepted 21 March 2014
Available online 3 April 2014

Keywords:
Alginate
Alginate gel
G-block
Lyase

ABSTRACT

Lyase-catalyzed degradation has been proposed as a more cell-friendly alternative to dissolution of alginate gels than using chelating agents. In this study, we investigated the effect of lyase specificity on degradation of alginate gels, including the effect of crosslinking ions with different affinity for the polymer. Degradation kinetics and products were analyzed. In particular, the degradation products were characterized using novel methods for alginate sequence determination by chromatography. Lyase-catalyzed gel disruption worked well for gels crosslinked with calcium, but was less effective when barium was included in the gel formulation. The importance of crosslinking of long G-blocks in maintaining the structural integrity of the gels was identified. The failure to degrade these long G-blocks, either due to protection of the G-blocks by strong ionic crosslinking or due to lack of lyase activity on G–G linkages, resulted in retained resistance to mechanical disruption of the gel.

1. Introduction

Alginate is a polysaccharide found in the cell walls of brown algae and secreted by some species of bacteria. It is a linear copolymer consisting of (1→4)-linked α-D-mannuronate (M) and its C-5 epimer β-L-guluronate (G) and may be considered as a block polymer, with homopolymeric G- and M-blocks, and MG-blocks with an alternating sequence (Haug, Larsen, & Smidsrod, 1966). It spontaneously forms a gel in the presence of divalent cations, of which particularly calcium or barium are used in biomedical applications (Grant, Morris, Rees, Smith, & Thom, 1973; Haug & Smidsrød, 1970). The ions bind to the respective block types with different affinity, and the cooperative binding between G-blocks in neighboring chains gives the strongest crosslinks (Haug & Smidsrød, 1970; Smidsrød, 1974; Smidsrød & Haug, 1972). Stokke, Smidsrød, Zanetti, Strand, and Skjåk-Bræk (1993) found that the G-block length necessary to form stable crosslinking junctions decreased with increasing affinity of the gelling ion. Alginate gelling can occur under physiological pH and temperature, enabling gentle entrapment of living cells or other sensitive materials (Smidsrød & Skjåk-Bræk, 1990). Alginate, with the exception of some structures found in microbial alginates (Stenvik et al., 2012), is generally recognized as non-toxic and non-immunogenic, and it is not known to interact specifically with cells. Alginate gels are widely explored in tissue engineering, as a delivery vehicle for drugs, and for basic biological studies (Augst, Kong, & Mooney, 2006; Krebs, Jeon, & Alsberg, 2009).

The gel often needs to be removed in order to release/recover the encapsulated cells or other encapsulated components that are not readily extracted through the gel matrix. The most common approach to dissolve the gels is by applying chelating agents such as EDTA or citrate, which act by sequestering the crosslinking ions in the gel, thereby dissolving it. Chelation works well for gels crosslinked with Ca^{2+}, but for ions with stronger affinity to alginate it is less effective. Some of this reduced efficacy can be compensated for by increasing EDTA/citrate concentrations, exposure time, and/or solution pH (Johnson et al., 2011). In biological studies, veering too far away from physiological conditions can damage any living cells present in the system or otherwise produce unwanted effects. The toxicity of extensive exposure to EDTA is well established (Cohen et al., 2011).

Lyase-catalyzed degradation of alginate gels is emerging as an increasingly popular alternative to chelation, and lyases have been effectively applied in a variety of studies, though mainly in applications where chelating agents are inefficient or inapplicable (Herlofsen, Kuchler, Melvik, & Brinchmann, 2011; Parrish, Siletz, Xu, Woodruff, & Shea, 2011; Sakai, Inagaki, Inamoto, & Taya, 2012; Shah et al., 2012). The data published so far do not identify any

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http://dx.doi.org/10.1016/j.carbpol.2014.03.076
0144-8617/

direct toxic effect on cells due to lyase exposure (Breguet, von Stockar, & Marison, 2007; Leslie et al., 2013).

Alginate lyases catalyze the degradation of alginate by cleavage of glycosidic linkages. All alginate lyases characterized catalyze the same kind of depolymerization, a beta-elimination which results in a new reducing end and a 4-deoxy-L-*erythro*-hex-4-enepyranosyluronate (Δ) at the non-reducing end (Gacesa, 1987). The lyases are commonly classified as M- or G-lyases according to their specificity, where M-lyases do not cleave GG-linkages, and G-lyases do not cleave MM-linkages. Most lyases have some activity on alternating blocks, usually with a preference for either MG or GM linkages. Although a lyase is classified as either M or G specific, it usually degrades alternating blocks as well and has in addition some residual activity towards the other homopolymer. Lyases with high activity on both homopolymers have also been isolated (Wong, Preston, & Schiller, 2000). Most lyases cleave the alginate endolytically, and demonstrate low activity on oligomers shorter than pentamer. During the course of the reaction, therefore, the accumulation of unsaturated di- and trimers and some tetramers is expected (Boyd & Turvey, 1977; Iwamoto et al., 2001).

In the few studies where alginate lyases have been applied on ionically crosslinked alginate, the choice of lyase has not been discussed, and the selection seems to have been based mostly on commercial availability. While the degradation products from lyase-catalyzed degradation of alginate in solution have been characterized to the point that lyases today are used as tools for alginate sequence determination (Aarstad, Tøndervik, Sletta, & Skjåk-Bræk, 2012), much less is known about their action pattern on alginate gels. In this study we used novel methods for alginate sequence determination by chromatography to study lyase-catalyzed degradation products of alginate gels. Although being an indirect method, this gives novel insight to gel structure by identifying sequences accessible and non-accessible for degradation in the gel. We believe that a better understanding of the parameters influencing lyase-catalyzed gel degradation will contribute in the optimization of the technique in current applications, as well as open up for a broader range of applications in the future. In this study, we investigated the effect of lyase specificity on gel degradation, including the effect of crosslinking ions with different affinity.

2. Materials and methods

2.1. Alginate beads

Alginate beads were produced using an electrostatic bead generator (NTNU dept. of physics, Norway) (Strand, Gåserød, Kulseng, Espevik, & Skjåk-Bræk, 2002). A 1.8% (w/v) UPLVG alginate (FMC Biopolymer, Lysaker, Norway), 0.3 M D-mannitol (VWR) solution was extruded through a 0.35 mm diameter nozzle into gelling buffer (10 mM HEPES, 0.14 M D-mannitol and either 50 mM $CaCl_2$, 50 mM $CaCl_2$ with 1 mM $BaCl_2$, or 20 mM $BaCl_2$). The beads were left in the gelling bath for 5–7 min after the last droplet in order to complete the gelling process. Finally, the beads were washed with 0.9% NaCl three times to remove unbound divalent ions and stored in phosphate-buffered saline (PBS) (beads gelled with barium) or PBS with 1 mM $CaCl_2$ (beads gelled with calcium alone) until use. Bead size was calculated as the average of 40 measurements.

2.2. Alginate lyases

Three lyases were tested: A commercially available lyase from *Flavobacterium multivorum* (Sigma Aldrich, St. Louis, MO, USA) ("G-lyase A"), another G-lyase from *Klebsiella aerogenes* specific for GG and GM linkages ("G-lyase B") produced recombinantly in *Escherichia coli* (Boyd & Turvey, 1977; Haugen, Kortner, & Larsen, 1990; Tøndervik et al., 2010), and a lyase from *Haliotis tuberculata* specific for MM and GM linkages ("M-lyase") (Haugen et al., 1990; Heyraud, ColinMorel, Girond, Richard, & Kloareg, 1996; Heyraud, Gey, et al., 1996). The lyases were dissolved in PBS and kept on ice until use.

2.3. Assessment of degradation by visual microscopy

A single layer of alginate beads was added to the wells of a 24-well plate. Lyase was added at a concentration of 0.1 or 1 U/mL in 0.5 mL PBS, and the samples were incubated at 37 °C for 2 h before inspection on a microscope. Beads in PBS without lyase were used as control. Average bead size after degradation was estimated based on measurement of 40 beads from each sample.

2.4. Degradation kinetics by UV spectrophotometry

The unsaturated uronate resulting from the lyase-catalyzed depolymerization reaction is a chromophore absorbing light at wavelengths in the region 230–240 nm, with a peak at 235 nm. Measurement of A_{235} during the course of the reaction can therefore be used to assess the activity of the lyases. The alginate beads were distributed evenly into the wells of a UV-transparent 96-well plate (Corning Inc.), and the supernatant (storage solution) was removed by pipetting from the bottom of the wells. Incubation buffer (PBS or RPMI 1640) was added, and the plate was then pre-incubated to reach a temperature of 37 °C. Finally, lyase was added to a final concentration of 0.1 U/mL, and the reaction was monitored by measuring A_{235} on a Tecan Infinite M200 Pro plate reader (Tecan, Männedorf, Switzerland) for 2 h.

2.5. Degradation of alginate beads for NMR and HPAEC-PAD analysis

Alginate beads were distributed evenly in glass bottles with PBS buffer and incubated with lyases at the same buffer:alginate:lyase ratio as for the UV spectrophotometry experiment, and then left at 37 °C for either 2 or 24 h. At the end of incubation, the reaction was stopped by heat denaturing of the enzyme (95 °C, 10 min). In order to completely dissolve the samples for downstream analysis of reaction products, EDTA (100 mM, pH 8) was added in excess. The samples were then dialyzed (Spectra/Por® dialysis membrane, MWCO 12-14,000, Spectrum Labs Inc.) against three changes of distilled water, three changes of 100 mM NaCl, and finally against distilled water until conductivity measurements read <0.1 μS. The samples were subsequently lyophilized and stored until further analysis. For the HPAEC-PAD analysis, the lyophilized samples previously degraded with G-lyase A were also degraded with M-lyase (0.1 U/mL, 24 h, 37 °C) in order to better visualize the G-block distribution.

2.6. ^1H NMR analysis. Samples were dissolved in D_2O (10 mg/mL)

Sodium 3-(trimethylsilyl)tetradeuteriopropionate (Sigma Aldrich) was used as an internal standard and triethylenetetraamine (Sigma Aldrich) was added to chelate residual divalent ions. ^1H NMR experiments were carried out on a Bruker Avance DPX 300 spectrometer with a 5 mm z-gradient DUL probe using Bruker XwinNMR v. 2.6 software. Spectra were recorded using a 30° flip angle at 90 °C. The data was analyzed using Bruker Topspin v. 3.0 software. The resonances in the anomeric region were assigned according to Grasdalen (Grasdalen, 1983) and Heyraud (Heyraud, ColinMorel, et al., 1996; Heyraud, Gey, et al., 1996).

2.7. High performance anionic exchange chromatography with pulse-aperiometric detection (HPAEC-PAD)

Samples were dissolved in distilled water (1 mg/mL) and analyzed on a Dionex DX-500 IC system (Dionex Corp., Sunnyvale, CA) comprised of an AS50 autosampler, an ED40 Electrochemical Detector with a non-disposable gold working electrode and a GP50 Gradient Pump. Data acquisition and analysis were performed using Chromeleon 6.7 software. Samples (25 μL) were separated as previously described (Tøndervik et al., 2010) at ambient temperature on a Dionex IonPac AS4A (4× 250 mm) anion-exchange column with an IonPac AG4A (4× 50 mm) guard column using a linear gradient of 8.75 mM sodium acetate/min in 100 mM sodium hydroxide. G-block (guluronic acid monomer fraction (F_G) > 0.97) and pure mannuronate partially degraded by M- and G-lyase respectively were employed as chromatographic standards. Non-gelled UPLVG alginate degraded with M-lyase was included as additional reference.

3. Results

3.1. Degradation of alginate beads assessed by visual microscopy

In all samples there were still gel beads present after 2 h of incubation with lyase (Fig. 1A). However, a reduction in size (Fig. 2) of the beads in some samples indicates that degradation has occurred. This was most evident in Ca-alginate beads incubated with G-lyase A, but was also seen for Ca/Ba gels with G-lyase A, as well as for Ca and Ca/Ba gels incubated with G-lyase B and M-lyase. In order to qualitatively assess the mechanical integrity of the beads after lyase exposure, the samples were pipetted vigorously several times and

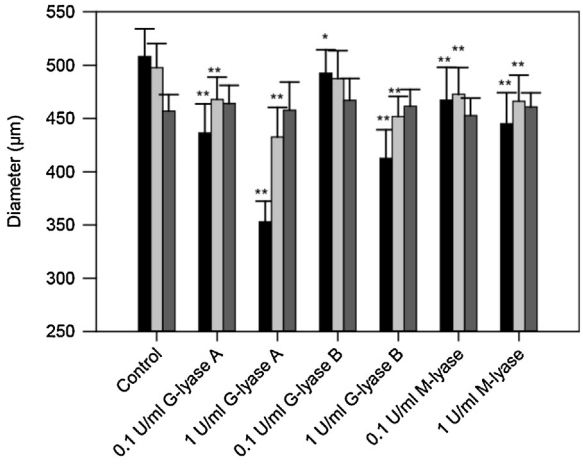

Fig. 2. Diameter of alginate beads after incubation with lyase for 2 h at 37 °C. Control is beads incubated in PBS without lyase. Ca-alginate (black), Ca/Ba-alginate (light gray), Ba-alginate (dark gray). Significant difference in size compared to control as assessed using unpaired *t*-test is indicated for $P < 0.001$ (**) and $P < 0.005$ (*). Error bars represent one standard deviation.

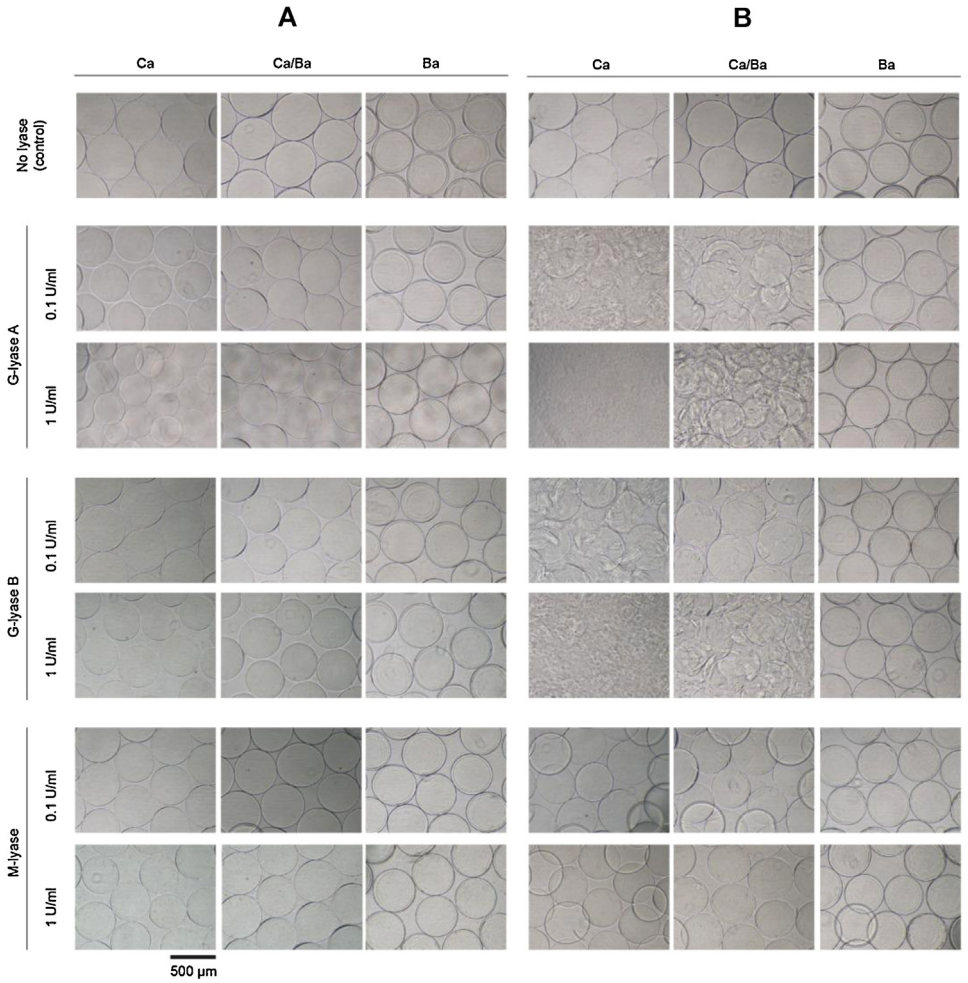

Fig. 1. Visual microscopy of lyase-degraded alginate beads. (A) Alginate beads incubated with lyase in PBS for 2 h at 37 °C. (B) The same sample after mechanical disruption by pipetting vigorously 15 times. Magnification: 4×.

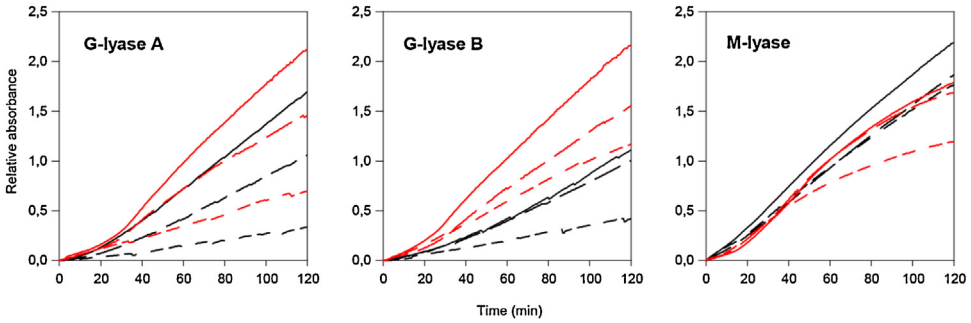

Fig. 3. Lyase-catalyzed degradation of alginate gels monitored by UV spectrophotometry. Time-course measurement of A_{235} for alginate beads degraded with the various lyases in PBS (black) and RPMI (red). Beads gelled with Ca^{2+} (solid line), Ca^{2+} and Ba^{2+} (long dots), and Ba^{2+} (short dots). The assay was repeated with similar results. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

inspected again (Fig. 1B). After exposure to mechanical stress, the difference became very apparent; all Ca-alginate beads exposed to G-lyase were disrupted, and the integrity of Ca/Ba-alginate beads incubated with G-lyase was also clearly weakened, while control samples as well as all Ba-alginate beads and beads exposed to M-lyase stayed intact.

3.2. Degradation kinetics by UV spectrophotometry

The degradation kinetics, as measured by the development in A_{235} during the degradation experiment, is presented in Fig. 3. For most conditions, there is an initial lag phase followed by an increase in degradation rate. This is most likely because the alginate beads are occupying only a fraction of the volume at the beginning of the incubation, but as degradation is progressing, alginate oligomers are released into the continuous phase, making it available to more of the lyase enzymes. For the two G-lyases, the degradation in RPMI proceeds faster than in PBS for all gelling ions. The degradation rate is also related to type of gelling ion, with a decreasing rate of degradation as the amount of barium is increased. These trends are not seen for the M-lyase. Mainly the G-blocks participate in ionic crosslinking, and as the M-lyase lacks the ability to cleave G–G linkages, it may be less sensitive to any direct effects of difference in crosslinking strength. None of the samples reach saturation in the timeframe studied, so this experiment does not provide any hints concerning differences in the final degree of degradation/plateau level.

3.3. Analysis of degradation products by ^1H NMR

The average degree of polymerization (DP_n) and monomer fractions in the degradation products calculated from the ^1H NMR spectra are shown in Table 1. Interpretation of the ^1H NMR spectra of lyase-degraded alginate (see supplementary data Figure S3) is made complicated by the presence of overlapping resonances, particularly for the reducing ends (Fig. 4). Due to partial overlapping of the resonances from reducing ends, M-$1_{red\beta}$ with GG-$1_{red\beta}$, and GG-$1_{red\beta}$ with MG-$1_{red\beta}$, the spectra were interpreted partly based on enzyme specificities from the literature. In order to calculate the total quantity of the reducing ends, empirical ratios (α/β) of 2.2 and 0.2 were used for mannuronate and guluronate, respectively (Heyraud, ColinMorel, et al., 1996; Heyraud, Gey, et al., 1996). It is important to keep in mind that the samples have been dialyzed after degradation, and that oligomers shorter than approximately 3–4 sugar units will have been removed. This leads to an underestimation of F_Δ and overestimation of DP_n. The average G-block length ($N_{G>1}$) is also commonly calculated from NMR. However, calculation of this parameter is based on assumptions that are only valid for long chains, and due to the low DP_n in degraded samples as well as the specificity of lyase-catalyzed degradation, these assumptions are not valid for the samples herein. The value of this parameter is also somewhat debated; Aarstad et al. recently reported evidence of an overall G-block distribution, and specifically the presence of very long G-blocks, that was not well reflected in the $N_{G>1}$ calculated from ^1H NMR spectra (Aarstad et al., 2012).

Table 1
Average degree of polymerization (DP_n) and monomer fractions of guluronate (F_G), mannuronate (F_M), and 4-deoxy-L-*erythro*-hex-4-enepyranosyluronate (F_Δ) calculated from ^1H NMR spectra recorded at 300 MHz of alginate beads incubated with lyase for 2 h at 37 °C. The samples were dialyzed prior to NMR.[a]

Lyase	Gelling ion	DP_n	F_G	F_M	F_Δ
Not degraded	–	–	0.67	0.33	–
G-lyase A (Sigma)	Not gelled	8	0.42	0.45	0.12
	Ca	16	0.57	0.37	0.06
	Ca/Ba	18	0.64	0.31	0.05
	Ba	24	0.67	0.29	0.04
G-lyase B	Not gelled	6	0.46	0.38	0.15
	Ca	13	0.60	0.33	0.08
	Ca/Ba	13	0.61	0.31	0.07
	Ba	28	0.66	0.30	0.04
M-lyase	Not gelled	17	0.76	0.19	0.06
	Ca	27	0.77	0.19	0.04
	Ca/Ba	29	0.76	0.20	0.03
	Ba	34	0.75	0.23	0.03

[a] Oligomers with a DP < 4 will have been removed, and the analyzed samples therefore enriched in non-suitable substrate for the lyase.

Overall, the degradation products for the two G-lyases look quite similar, while the M-lyase stands out, which is expected based on their known specificities (Supplementary Table S1). The two former

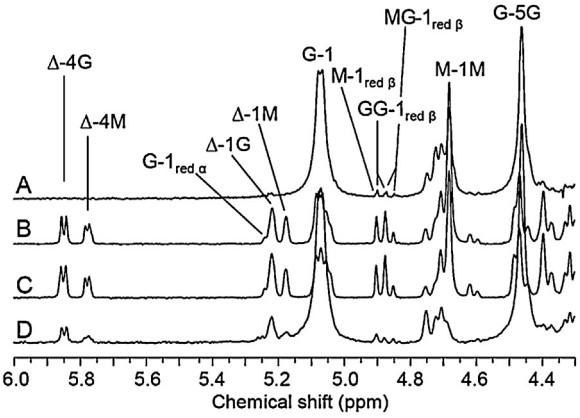

Fig. 4. ^1H NMR (300 MHz, 90 °C) spectra of alginate in solution. (A) No lyase. (B) G-lyase A. (C) G-lyase B. (D) M-lyase. Samples were incubated with 0.1 U/mL lyase at 37 °C for 2 h and then dialyzed and lyophilized before NMR.

degradation produce a similar DP_n for each of the respective gel formulations, and F_G is decreasing in the order Ba > Ca/Ba > Ca > Not gelled. The M-lyase samples are the least degraded overall, something that at least partly should be attributed to its low activity on G-blocks, as the alginate used contains 67% guluronate and therefore less suitable substrate for the M-lyase than for the lyases with G–G activity. The low activity on G-blocks is also confirmed by the relative increase in F_G, indicating that mostly M residues have been converted into Δ and/or removed in dialysis. In summary, these observations are in harmony with what is known about the lyase activities from literature.

3.4. Analysis of degradation products by HPAEC-PAD

HPAEC-PAD chromatograms for gels degraded with G-lyase A are shown in Fig. 5. Differences between the gel fraction samples can be seen for high retention times (>75 min), where longer chains are present in the Ba-gels than in the Ca and Ca/Ba gels. For both the Ca/Ba- and the Ba-gels, the composition of the gel fraction seems to be maintained from 2 to 24 h of degradation, however the spectra of the soluble fractions indicates further degradation. After 24 h, no gel was left in the sample gelled with calcium alone. Detailed analysis of the degradation products was difficult due to the presence of oligomers with heterogeneous composition together with the oligomers of different block types, as the strong dependence of composition on retention times, exemplified by reference chromatograms for pure G-blocks and M-blocks (Fig. 5B), results in co-elution for DP ≥ 5.

Recovery yields were measured after dialysis for the respective fractions (Supplementary Table 2). For the Ca beads, about 30% of the alginate initially added to the reaction was left in the gel fraction after 2 h. At 24 h there was no gel fraction left, and most of the alginate was gone in dialysis, indicating the presence of a large amount of lyase end-products. For the Ba beads, most of the alginate added was recovered, and around 20% was found in the soluble fraction after 2 h. No change was seen with extended incubation. For the Ca/Ba beads the recovery was reduced between 2 and 24 h, and at the end of incubation around 40% of the alginate was left in the gel fraction.

G-blocks are the main contributors to ionic crosslinking, and it is of particular interest to resolve whether their close association with the gelling ions allows polymer cleavage by the lyases. In order to better visualize the G-blocks in the samples, the samples were further degraded with M-lyase, which degrades MG- and M-blocks while leaving G-blocks intact (Supplementary Table S1). The resulting chromatograms are shown in Fig. 6. Overall, both of the barium-containing beads show very similar G-block distribution for their respective 2- and 24-h samples, while the beads gelled with calcium alone shows a significantly shorter G-block length. A broad peak similar to the one seen in the G-block reference (Fig. 5B) is found for all gel fraction samples, and a slightly higher retention time than the reference indicates the presence of very long G-blocks. The degradation of gel fraction of Ba gels by M-lyase resulted in reduced retention time and a change in shape of the broad peak observed at 70–80 min after G-lyase degradation, now showing similar patterns both in shape and retention time as the Ca- and Ca/Ba gels. This indicates the presence of some M in or between the crosslinking zones of the Ba-gels that is not initially degraded by the G-lyase.

All samples gelled with barium also contained a varying amount of intermediate and shorter G-block oligomers in the gel fraction. The Ba-alginate gel contained both small and intermediate G-blocks and overall a G-block distribution very similar to the starting material. In comparison, there were significantly less of the shorter G-blocks in the gel fraction for Ca/Ba-alginate beads. At least part of this difference may be accounted for by the short G-blocks that are present in the soluble fraction for Ca/Ba beads, but not for Ba beads.

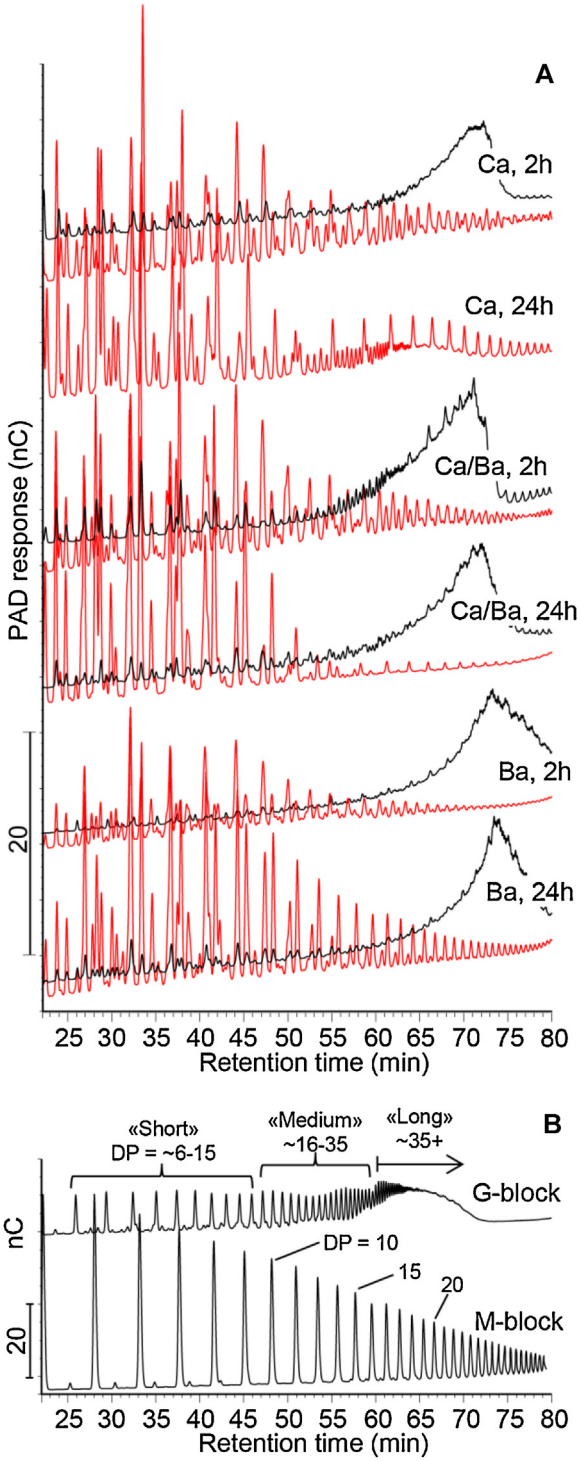

Fig. 5. HPAEC-PAD chromatograms. (A) Ca-, Ca/Ba-, and Ba-beads degraded with G-lyase A gel fraction (black) and soluble fraction (red). No gel fraction sample was left for the Ca-beads after 24 h of incubation. (B) M-block and G-block standards. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

For the soluble fractions, the general picture is that they contain a small amount of relatively short G-blocks, but mainly lyase end-products (dimer–hexamer). There is no clear difference between the 2-h and 24-h samples for the Ca or Ca/Ba beads. For the beads gelled with both calcium and barium, there are some G-blocks of a

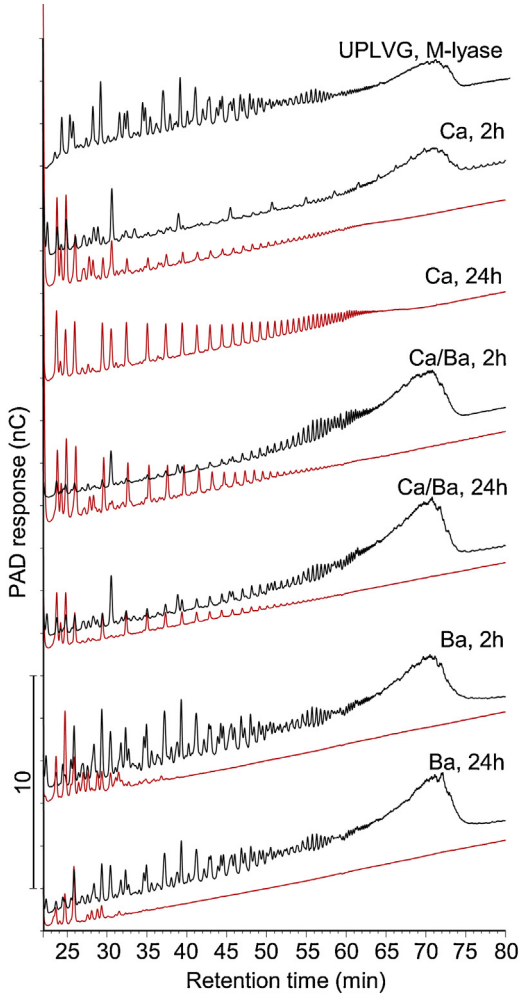

Fig. 6. HPAEC-PAD results from Ca-, Ca/Ba-, and Ba-beads degraded with G-lyase A for 2 or 24 h, followed by incubation of solubilized fractions with M-lyase for 24 h. Black: Gel fraction. Red: Soluble fraction. No gel fraction sample was left for the Ca-beads after 24 h of incubation with G-lyase A. UPLVG alginate degraded with M-lyase (top) shows the G-block distribution in the original polymer. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

small-medium length that are not seen in the corresponding samples for barium beads.

4. Discussion

Alginate lyases have in several studies been applied in order to degrade alginate gels (Breguet et al., 2007; Herlofsen et al., 2011; Sakai et al., 2012; Shah et al., 2012). However, these studies have generally been instrumental and limited to degradation of calcium-alginate gels or covalently crosslinked alginates, while variables such as lyase specificity and strength of ionic crosslinking have not been studied in any detail. In this study, the degradation of three different alginate gels using lyases with different specificity was studied, and the degradation products were analyzed using qualitative and quantitative techniques. Parameters such as degradation temperature and buffers were selected in order to keep the study relevant to applications involving mammalian cells, and therefore deviate somewhat from the optimal conditions for lyase activity as they are reported in the literature. A commercially available lyase (G-lyase A) was included due to its availability to the community and its past application in several published studies.

4.1. Effect of lyase specificity

It was found that efficient gel disruption requires a lyase with high activity on G–G linkages. Degradation with M-lyase did not result in dissolved gels for any of the conditions tested for the high G alginate used in this study. Interestingly, beads incubated with G-lyase were much less resistant to mechanical stress (Fig. 1) than the corresponding samples incubated with M-lyase, even though G-lyase B and M-lyase showed a similar reduction in bead size (Fig. 2), and the rate of polymer cleavage for M-lyase is comparable to the kinetics measured for G-lyase (Fig. 3). The alginate used in this study contains a relatively large amount of long G-blocks (Fig. 6). Such blocks may interact with several adjacent alginate chains to form multiple crosslinking zones, acting as a backbone holding the structure together. The M-lyase has only a negligible activity on G-blocks (Supplementary Table 1), and we hypothesize that this inability to cleave the backbone of the gel network is the main reason for its low efficacy in gel disruption. The lyases were also briefly tested on beads made with high M alginate ($F_M = 0.64$), and although M-lyase worked better on this more suitable substrate, the G-lyases were still far more effective at gel disruption (data not shown).

4.2. Effect of ionic crosslinking strength

Beads gelled with barium ions, both alone and mixed with calcium, were much more resistant to lyase-catalyzed degradation than beads gelled with calcium alone. The Ba-alginate beads were the smallest in this study, and the more compact gel network may lower the permeability and thereby limit diffusion of enzyme into the beads. In a study using similar bead formulations, Mørch, Donati, Strand, and Skjåk-Bræk (2006) concluded that Ba-alginate beads were permeable to IgG (~150 kD), although less so than Ca- or Ca/Ba-alginates. The size of the alginate lyases used in this study is around 30–40 kDa, much smaller than IgG, and permeability is thus not expected to significantly limit diffusion of the lyases into Ba-alginate beads. The difference in degradability therefore seems to be related to the crosslinking strength, with crosslinking zones of barium bound to G-blocks being protected from lyase-catalyzed degradation, while the interaction between calcium and G-blocks is not strong (or stable) enough to protect the polymer participating in crosslinking zones. Phosphate has a chelating effect that may contribute to destabilize the crosslinking zones, particularly for the calcium beads, but our results indicate that this contribution is not large enough to on its own influence bead stability. The addition of a small amount of barium to the same formulation as is used for calcium-alginate beads does stabilize the beads somewhat, but as shown in Fig. 1B, the mechanical stability of the Ca/Ba-alginate beads is severely reduced after incubation with lyase. The G-block distribution in the beads after lyase degradation (Fig. 6) indicates that while the Ba beads retains a distribution similar to the starting material, the Ca/Ba beads show a reduction in the amount of short (DP < 15) G-blocks. This is in accordance with what is previously known from literature, where it has been reported that the G-block length necessary for stable crosslinking decreases with increasing affinity of the gelling ion (Stokke et al., 1993). The results herein indicate that while a small amount of barium does not seem to significantly influence the length of the G-blocks participating in crosslinking in Ca/Ba beads compared to Ca beads, it stabilizes the crosslinking zones enough to protect them from lyase-catalyzed degradation.

4.3. Conclusion and future perspectives

In summary, lyase-catalyzed gel disruption works well for gels crosslinked with calcium, but is less effective when a gelling ion

with higher affinity is used. The importance of long G-blocks in maintaining the structural integrity of the gels was identified, and the failure to degrade these long G-blocks, either due to protection of the long G-blocks by strong ionic crosslinking or due to lack of lyase activity on G–G linkages, resulted in retained resistance to mechanical disruption of the gel.

Future studies should investigate combinations of lyase and chelating agents to test whether the two strategies may work in synergy to efficiently disrupt Ba-alginate beads under more physiologically appropriate conditions than required for chelation alone. Also, the apparent protection of crosslinking zones in strong/stable gels may also be exploited to further characterize differences in crosslinking patterns in different ionically crosslinked alginate gels.

Acknowledgements

The authors thank Wenche I. Strand for performing ^{1}H NMR analysis. This work was supported by the Norwegian Research Council Grant #182695-I40. B.L.S. acknowledges the support from the Liaison Committee between the Central Norway Regional Health Authority (RHA) and the Norwegian University of Science and Technology (NTNU).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.03.076.

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