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Molecular mass distribution of sodium alginate by high-performance size-exclusion chromatography

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Abstract

A sensitive high-performance size-exclusion chromatography (HPSEC) method with simple UV detection was developed for the molecular mass analysis of sodium alginate. It was used to evaluate alginates of varying molecular mass and the results were compared with the viscosity measurements. This HPSEC method was sensitive to serve as the stability indicating method for alginate after storage under different conditions. The information of relative molecular mass distribution of alginate was provided with reference to pullulan molecular mass standards. The comparison of the HPSEC chromatograms of alginate, pullulan and dextran revealed the effect of chemical composition of a polysaccharide and its effect on apparent molecular mass distribution.

Keywords: Molecular mass distribution; Sodium alginate; Dextran; Pullulan; Polysaccharides

1. Introduction

Sodium alginate is a member of polysaccharide family. It is isolated from seaweed and is made up of unbranched copolymers of 1–4 linked D-mannuronic acid and L-guluronic acid [1]. The ratio of D-mannuronate/L-guluronate can be determined by nuclear magnetic resonance (NMR) [2,3] or high-performance liquid chromatography (HPLC) [4]. A solution of sodium alginate can form gels by intermolecular hydrogen bonding and ionic crosslinking between the carboxyl groups of alginate and multivalent metal ions [5–7]. In addition, alginate can be chemically modified to induce covalent crosslinking [8]. Because of its gelling ability, stabilizing properties and its high viscosity, alginate is widely used in the food [9–11] and pharmaceutical industries [12–14]. For example, capsules can be made from combining alginate with calcium and used for encapsulation of cells [15,16] or drug delivery [12]. We have been using alginate for encapsulation of islet cells to treat patients with type I diabetes [17]. Because the behavior of alginate is strongly dependent on its molecular size and intrinsic viscosity, it is important to develop a simple but effective method for the molecular mass characterization and quality control for alginate especially for pharmaceutical use.

Several ways of measuring molecular mass of polysaccharides exist, such as size-exclusion chromatography (SEC), light scattering through Zimm's plot or Mark–Houwink fitting, sedimentation analysis in the analytical ultracentrifuge, and intrinsic viscosity. This subject was reviewed and discussed

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by Harding et al. [18]. The SEC method provides information on relative or absolute molecular mass distribution depending on the standards used for calibration. Pullulan and dextran can be served as standards to determine the relative molecular mass of alginate. Universal size calibration with pullulan and dextran has been reported by Fishman et al. [19]. The light scattering method and the analytical ultracentrifuge method are reliable and provide an absolute value of molecular mass. The intrinsic viscosity method is easy to operate however it relies on the measurements of viscosity at low sample concentration, and reasonable error may be introduced in molecular mass determination because of the difficulties in accurate measurements of low viscosity samples. Recently SEC–MALLS and SEC–AUC methods were developed which combined SEC with multi-angle laser light scattering detection (MALLS) and the analytical ultracentrifuge technique (AUC), respectively, to measure molecular mass distribution (MMD) of carbohydrate polymers [20,21]. These methods provide absolute value of molecular mass and are not dependent on the use of calibration standards. Their application to sodium alginate and dextran was discussed by Horton et al. [22] and Ball and co-workers [23,24].

It has been determined that the SEC–MALLS and SEC–AUC methods are very effective in measuring molecular mass of sodium alginate. Unfortunately the instruments required for these methods are not available in our laboratory. We therefore applied a basic HPLC system to develop a simple and effective SEC method for quality control and stability analysis of sodium alginate. The SEC methods have been widely used for determining MMD of polymers and proteins [25,26], however, limited studies have been done on alginate [27–30]. The effect of ionic strength of mobile phase on the elution of alginate was studied by Fujihara and Nagumo [27], Yoshio et al. [29], and Gan and Liu [30]. The MMD of alginate and its intrinsic viscosity distribution were derived by Hoagland et al. [28] after universal calibration with pullulan standards. The comparison of the molecular mass of alginate by SEC and viscosity measurements was made by Fujihara and Nagumo [27].

In this paper a more detailed discussion of the development of a simple, rapid but effective and sensitive HPSEC method is presented. The results on an alginate stability study are reported. It is demonstrated that this method can be used to not only provide information on MMD of alginate, but also serve as a quality control and stability indicating method. The strong dependence of SEC retention on chemical conformation is illustrated by comparing the chromatograms of alginate with other polysaccharides.

2. Experimental

2.1. Sample materials

Sodium alginate samples were obtained from A/S Drammen, Protan, Norway. The narrow MMD standards of pullulan were purchased from Polymer Labs., MA, USA. The dextran standards were from Polysciences, PA, USA. Samples were prepared in deionized (DI) water for the HPSEC analysis.

2.2. HPSEC

The HPLC system was equipped with the following instruments from Waters Corporation: 600 solvent delivery system, 717 plus autosampler, 996 photodiode array (PDA) detector, and 410 differential refractometer (RI detector). The PDA detector and the RI detector were connected in series. It took less than 0.05 min for the mobile phase to flow from the PDA detector to the RI detector. Alginate was detected at 210 nm by the PDA detector, whereas pullulan and dextran were detected by the refractive index detector at 33°C. Data were collected and analyzed by Waters Millenium software. TSK-GEL size-exclusion columns were used in the method development of alginate. The G5000PW_{XL} (30×7.8 mm, 10 μm particle size, 1000 Å pore size) and G6000PW_{XL} (30×7.8 mm, 13 μm particle size, >1000 Å pore size) columns were connected in series. DI water or buffer solution was filtered, degassed and then used as mobile phase at a flow-rate of 0.7 ml/min. The buffer solution was 0.1 *M* monobasic ammonium phosphate (Fisher) solution with pH adjusted to 4.0 by phosphoric acid (Aldrich). Sodium chloride (Aldrich) was added (0.3 *M*) to

increase the ionic strength of the mobile phase. Sodium alginate was dissolved in water at a concentration of 0.2 to 0.5%. Injection volumes were 15 μl.

2.3. Viscosity measurement

The viscosity of sodium alginate as a function of its concentration was measured to evaluate different alginate samples, the results were compared to those obtained by the HPSEC method. The viscosity measurements were obtained by using a spindle cone Rheometer (Brookfield Digital Rheometer DV-III). The Rheometer was equipped with two spindles: CP-41 and CP-52 which measured solutions in the range of 0.25 to 1.5% and 2 to 2.5%, respectively. A solution of 2.5% alginate was prepared by dissolving sodium alginate in DI water, followed by dilutions for 2, 1.5, 1, 0.5, 0.25% solutions.

2.4. Forced degradation and stability study

A forced degradation study and a short-term (one month) accelerated stability study were performed to see if the method was sensitive to the change in the MMD of alginate. In the forced degradation study alginate was degraded by acid hydrolysis. A 0.5% sodium alginate solution was first adjusted to pH 5.0 by 0.05 *M* HCl and then heated to 100°C for 1 h in an oven. The solution was cooled down to room temperature, adjusted to pH 4.0, and again heated to 100°C for 1 h. The degraded alginate sample was cooled down to room temperature and neutralized to pH 7.0 by 0.05 *M* NaOH. No aggregation was observed at this stage. A small amount of sample was withdrawn at this stage for the analysis of MMD by HPSEC and viscosity measurements. The rest of the sample was further degraded by the same procedure and then analyzed for the MMD.

Alginate stability samples were prepared at different pH. Sodium alginate was dissolved in pH 4.5 buffer solution, DI water, and pH 9.1 buffer solution, respectively, to ~0.25%. The samples were then aliquoted into three bottles and put in a 55°C oven. Samples at each pH were retrieved at one week, three weeks, and four weeks for the analysis of the MMD. In the above samples, no aggregation was observed.

3. Results and discussion

3.1. HPSEC method development

Polysaccharides generally have poor UV sensitivity, therefore RI detectors are commonly used in chromatographic analysis. However it is possible to apply UV detection for alginate since it shows reasonable far UV sensitivity due to the carboxylic functional group in the uronic acid (see Fig. 1). Compared to a RI detector, UV detectors are much easier to operate, do not require stringent temperature control, have less interference from other components in mobile phase and less sensitive to the noise caused by temperature fluctuation and pump pulses. Fig. 2 shows a comparison of alginate chromatograms by UV and RI detection. The two chromatograms have similar peak shape and retention time, however, UV detection gives much higher signal-to-noise ratio.

Since alginate is known to have very broad MMD, two TSK-GEL columns ($G5000PW_{XL}$ and $G6000PW_{XL}$) were combined for the MMD analysis. Since no alginate molecular mass standards are commercially available, the columns were calibrated with the pullulan molecular mass standards. Pullulan consists of glucose residues, which are joined by α-1,4 linkage and branched through α-1,6 linkage. Molecular mass standards of dextran, which consists of glucose residues joined exclusively by α-1,6 linkage, were also used as a comparison. The chemical structures of alginate, pullulan and dextran are compared in Fig. 3. The chromatograms of pullulan and dextran standards are overlaid respectively and compared with an alginate in Fig. 4. A reasonable separation was achieved for pullulan standards with molecular mass ranging from 5000 to 800 000, and for dextran from 10 000 to 600 000. The calibration curve for pullulan was plotted as the molecular mass on a log scale against the retention time. These data were best fitted by a linear regression with a slope of −0.2011 and intercept of 10.083. The correlation coefficient (R^2) is 0.9907. Although the absolute MMD of alginate cannot be determined by this HPSEC method without the narrow molecular mass distribution alginate standards, the information of the relative or apparent MMD can be provided. With reference to pullulan,

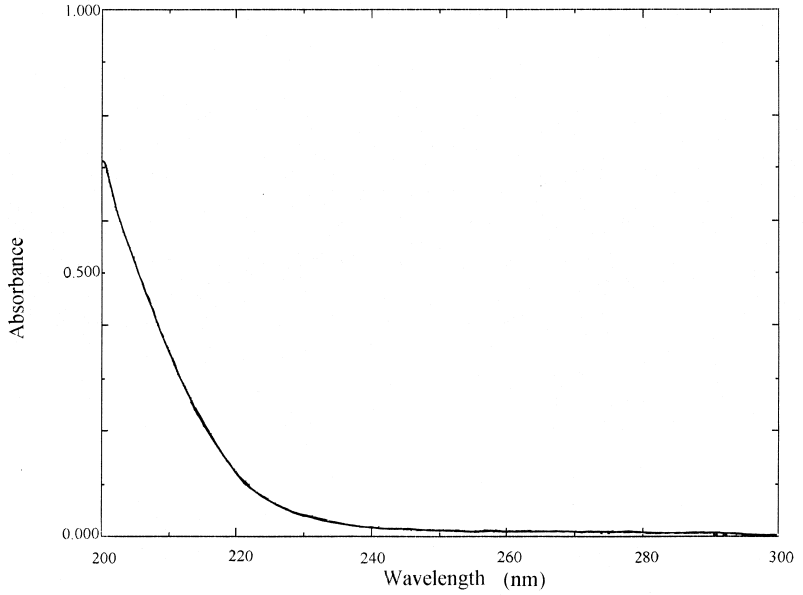

Fig. 1. Absorption spectrum of alginate (0.05% in 1 cm pathlength cell) in DI water. The spectrophotometer was from Shimazu (UV-1601PC).

the relative molecular mass of the above alginate sample at its peak maximum was predicted to be 872 000.

Sodium alginate is an ionic polymer. The inter- and intra-molecular ionic repulsion tends to expand its size, which may minimize its gel permeation retention. In addition, the sorbent of the gel columns contains some negative charges [29]. The ionic repulsion between the anionic alginate molecules and the sorbent may cause early elution. Therefore, to achieve the optimum retention, it is very important to adjust pH and ionic strength of mobile phase. The pK_a values of mannuronic and guluronic acid are 3.38 and 3.65, respectively [31]. It is known that alginates can be precipitated at low pH. In order to increase the distribution of non-ionized molecules in sodium alginate and while maintaining solubility, the pH of the mobile phase was adjusted to 4.0 by adding phosphoric acid to 0.1 *M* ammonium phosphate solution. Sodium chloride was also added to 0.3 *M* to increase the ionic strength of the mobile phase. Fig. 5 illustrates the importance of the mobile phase adjustment to alginate retention. When DI water was used as mobile phase, chromatograms of abnormal shapes were observed and the reproducibility of the chromatograms was poor. The relative standard deviation of the peak area for the three consecutive injections was 12%. The peak area increased for the later injection (area f>area e>area d). This might indicate that there was some alginate sample residue absorbed on the column when DI water was used as mobile phase. In contrast reasonable peak shape was observed with the maximum of the alginate peak shifted from 17.5 min to 20.6 min when the low pH and high ionic strength buffer was used as mobile phase. The reproducibility of the chromatograms was greatly improved with a 3% relative standard deviation for the three consecutive injections. Similar observation was reported by Fujihara and Nagumo [27], Yoshio et al. [29], and Gan and Liu [30] on the effect of ionic strength of mobile phase on gel permeation retention of ionic polymers.

3.2. Analysis of alginates with different molecular masses

The HPSEC method was used for the analysis of eight sodium alginate samples. Although all of the alginate samples have a very broad MMD, their chromatograms enabled us to categorize them into

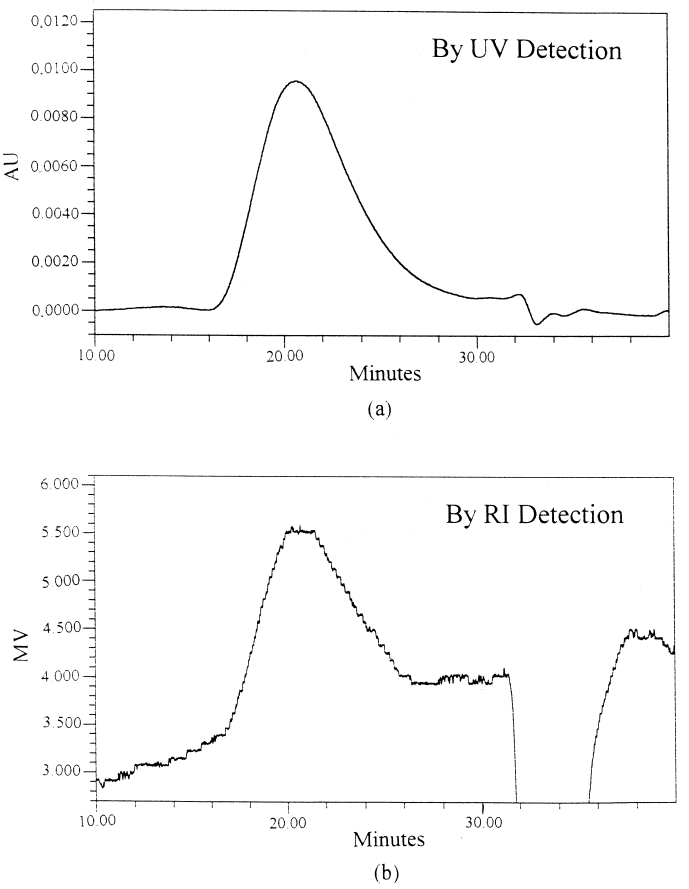

Fig. 2. Chromatograms of alginate. UV detection at 210 nm (a) versus RI detection (b). The two chromatograms are obtained by using the same sample, mobile phase and injection volume.

four groups from low to high MMD. The chromatograms representing each group are overlaid in Fig. 6. This observation was verified by the viscosity data. The viscosity of the eight alginate samples was measured as a function of their concentration. The viscosity data can be categorized into the same four groups as determined by the HPSEC method with group a representing the highest and group d representing the lowest MMD (see Fig. 7). The consistency in the analysis results by the two methods demonstrates the efficiency and reliability of the HPSEC method for the determination of MMD of alginate. The relative MMD of alginate at its peak maximum was estimated as 913 000, 691 000, 397 000 and 274 000, respectively by reference to the molecular mass calibration curve of pullulan.

3.3. Forced degradation and stability study

In the forced degradation study, alginate was degraded twice by acid hydrolysis as described in Experimental. The degraded samples were tested by the HPSEC method and the chromatograms are shown in Fig. 8. The peak shape looked similar before and after degradation. However the elution time of alginate at the peak maximum was shifted from 20.6 min to 26.4 min after the first degradation and to 29.0 min after second degradation. The peak height for the degraded samples increased because the final volume of the degraded samples was significantly decreased due to water evaporation in the hydrolysis process. The pronounced shift in the elution time indicates that the HPSEC method is

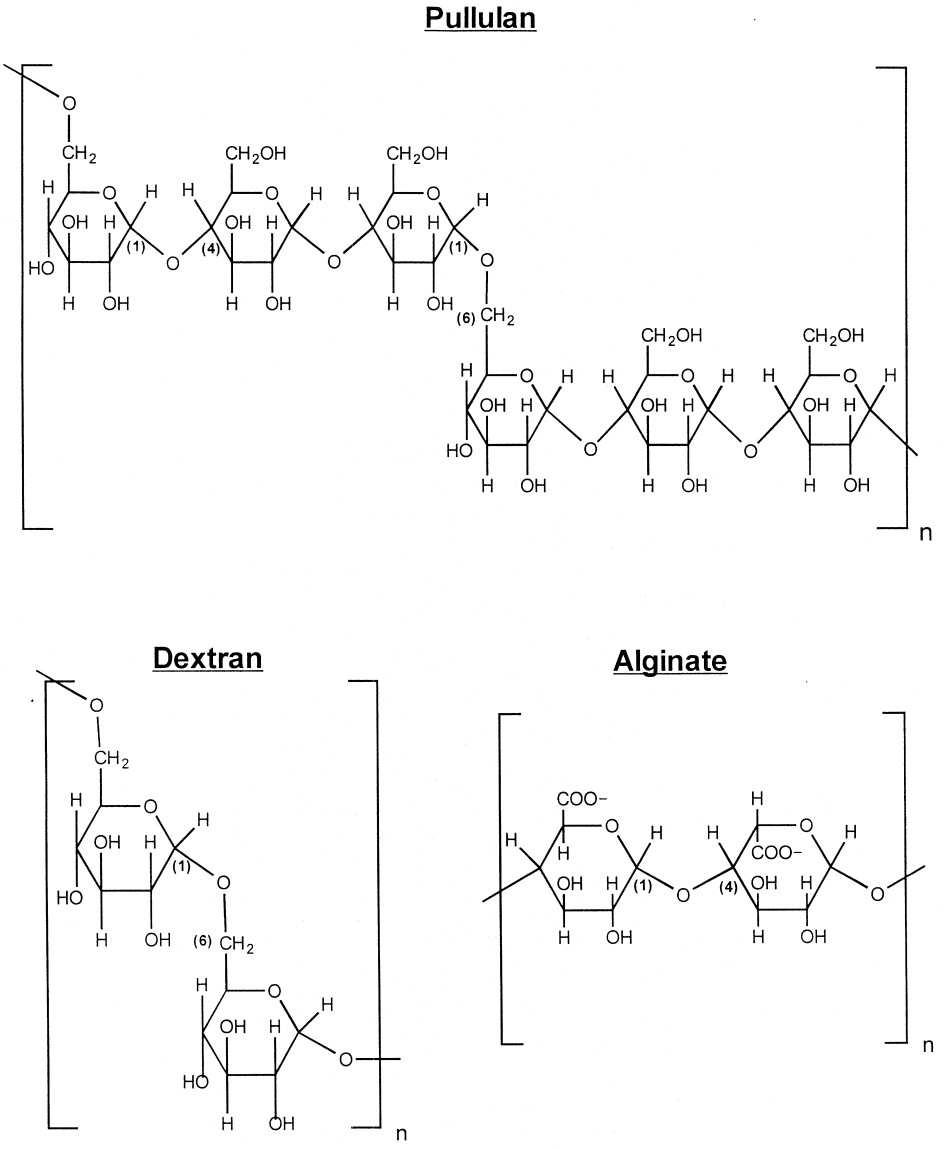

Fig. 3. Chemical structures of pullulan, dextran and alginate.

sensitive to the change in the MMD of alginate. The change of the relative molecular mass of alginate at its peak maximum was estimated to be from 872 000 to 59 000 after the first degradation and to 18 000 after the second degradation. This observation of degradation by the HPSEC method was also supported by the viscosity measurements. The viscosity value of alginate dropped from 30.8 cP to <7 cP after first degradation. For the second degradation, the viscosity value was too low to be measured by our instruments.

A stability study of alginate was conducted at 55°C for a month. Alginate samples were prepared in different solutions with concentration at ~0.25% as

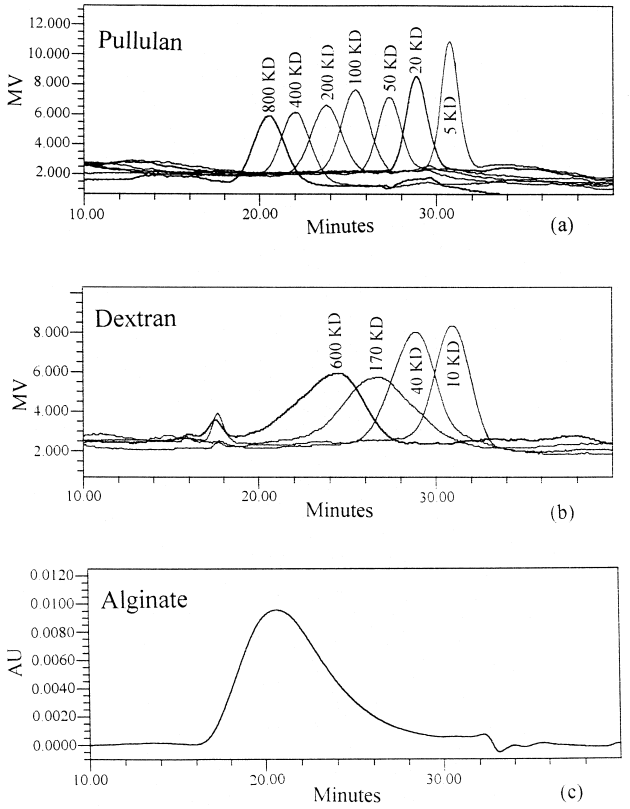

Fig. 4. Overlay of chromatograms of molecular mass standards of pullulan (a) and dextran (b) with DI water as mobile phase and RI detection, and chromatogram of alginate (c) with pH 4 buffer solution as mobile phase and UV detection. Same retention time and peak shape are observed for pullulan and dextran with pH 4 buffer solution as mobile phase. kD=Kilodalton.

described in Experimental. The samples retrieved at each time point (no aggregation was found) were analyzed by HPSEC. The results are demonstrated in Fig. 9. After storage of alginate in DI water or pH 9 solution for a month no obvious change in the MMD was observed. Although at week 3 and week 4 there was a slight difference in the peak area between the pH 7 and pH 9 samples, the retention time at their peak maximum was very similar to that at time zero. This indicates that there was no significant degradation in the pH 7 and pH 9 samples. Unlike the pH 7 and pH 9 samples, the pH 4.5 sample showed a significant change in the retention time even after one week of storage. No aggregation was observed in the samples. In addition, the mobile phase was adjusted to low pH and high ionic strength, which minimized the electrostatic effect on the SEC retention. Therefore the significant change observed in its chromatograms was most likely caused by degradation. The retention time of alginate at the peak maximum shifted from 20.5 min to 24.0 min after one week, to 26.60 min after three weeks, and to 28.7 min after one month. By reference to pullulan the relative molecular mass of alginate at its peak maximum was estimated to change from 913 000 to 181 000 after one week, to 54 000 after three weeks, and to 20 000 after one month storage in the pH 4.5 solution. Alginate is not stable in pH 4.5 solution possibly due to the hydrolysis of the glycosidic linkage.

The instruments equipped in our laboratory for the viscosity analysis can not provide an accurate measurement when the viscosity is below 7 cP. The alginate stability samples in our study was prepared

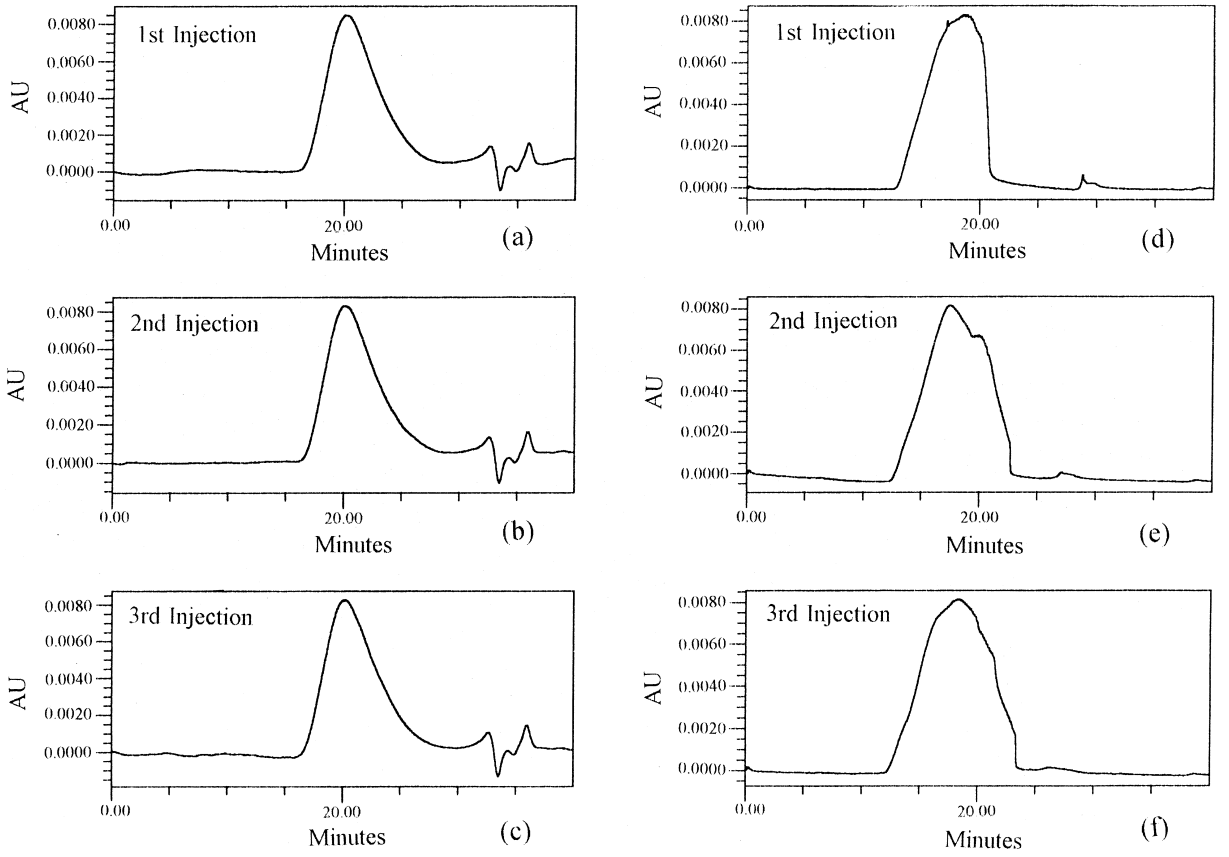

Fig. 5. Comparison of chromatograms of consecutive injections of alginate with pH 4 buffer solution as mobile phase (a–c) or DI water as mobile phase (d–f) showing abnormal peak shape and poor reproducibility.

at ~0.25%. The deviation in the viscosity measurement was too big to provide any information on the stability. However Fig. 9 demonstrates that even at low concentration the HPSEC method is still sensitive to the degradation of the stability samples.

3.4. Effect of chemical composition on SEC retention

SEC is a chromatographic method that separates molecules based on their size. The SEC elution is strongly dependent on the chemical composition of the sample molecules. Alginate, pullulan and dextran all belong to the polysaccharide family and they consist of similar base units (uronic acid for alginate, and glucose for pullulan and dextran). The uronic acid chain in alginate is formed by α-1,4 linkages. The glucose chain in pullulan is linked by α-1,4 linkages and branched by α-1,6 linkages, while in dextran is linked exclusively by α-1,6 linkages (see Fig. 3). The different chain units and linkages may introduce significant difference in their chemical conformation resulting in different apparent molecular mass. Fig. 10 shows an overlay of the chromatograms of alginate, pullulan and dextran. Dextran has an average molecular mass of 600 000, however, it was eluted after pullulan with molecular mass of 400 000. This indicates that the size of dextran is smaller than that of pullulan. Since alginate contains negative charges the ionic repulsion may lead to a much more expanded and inflexible shape than pullulan and dextran. Therefore at the same molecular mass alginate is likely eluted before pullulan and dextran. In other words, at a given molecular mass

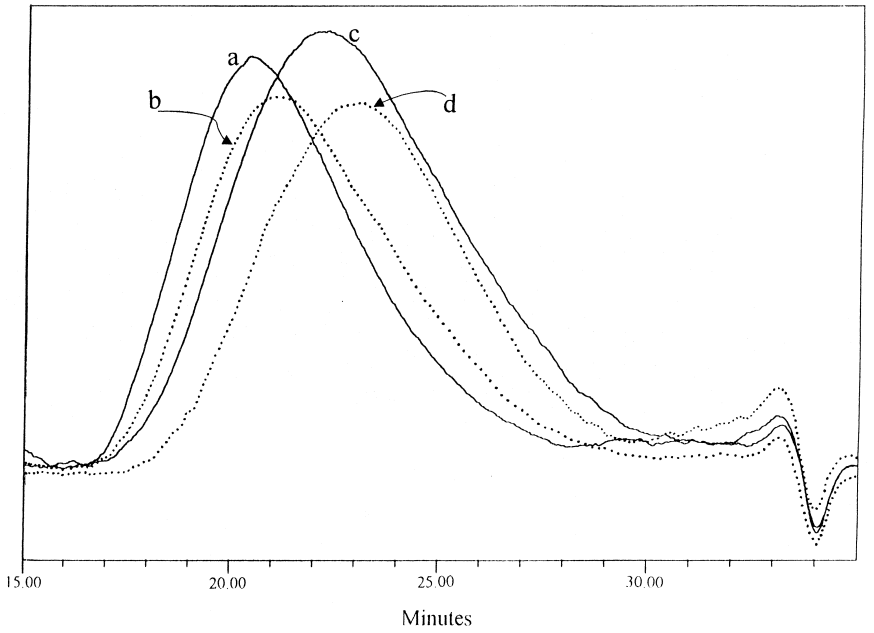

Fig. 6. Chromatograms of alginate a, b, c and d with varying molecular mass at 913 000, 691 000, 397 000 and 274 000, respectively (relative to pullulan standards).

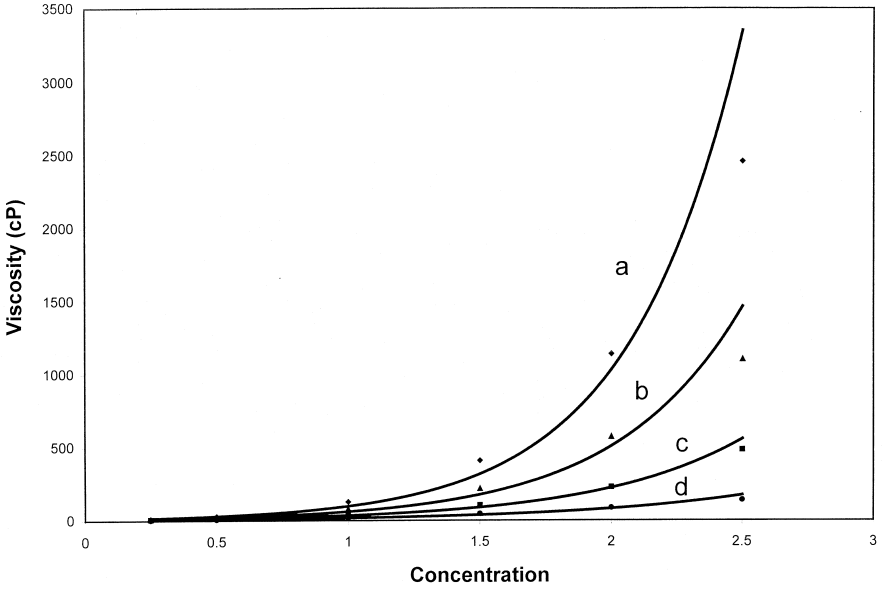

Fig. 7. Plots of concentration versus viscosity for alginate a, b, c and d. Data were best fitted to the exponential form. For sample a, $y=9.232\ \exp(2.3577x)$, for sample b, $y=7.2874\ \exp(2.1211x)$, for sample c, $y=5.6031\ \exp(1.8387x)$, and for sample d, $y=3.7782\ \exp(1.5253x)$.

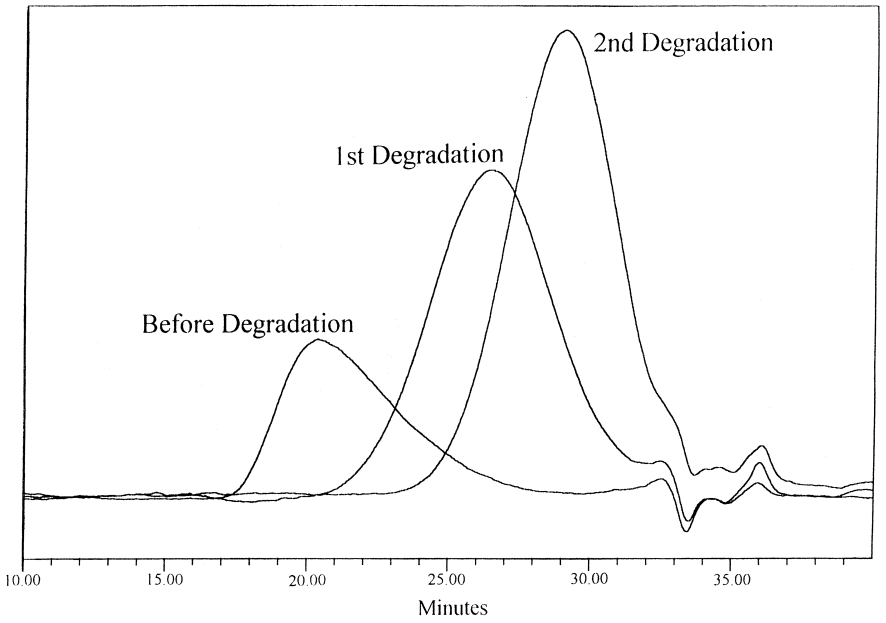

Fig. 8. Chromatograms of alginate before and after first degradation (heated at pH 4.5 and 100°C for 2 h) and second degradation (further degraded by the same procedure).

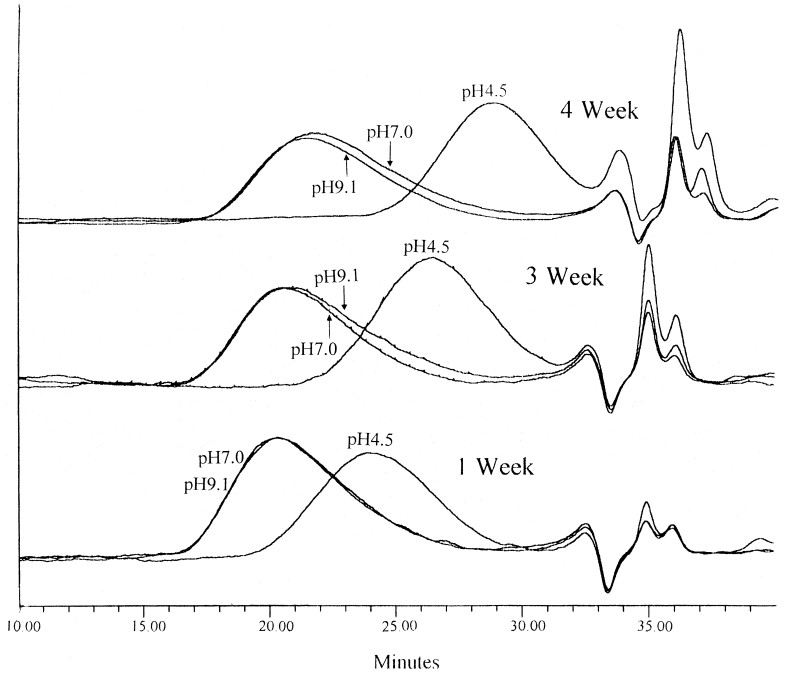

Fig. 9. Chromatograms of alginate in pH 4.5, pH 7.0 and pH 9.1 solution after storage at 55°C for one week, three weeks, and four weeks.

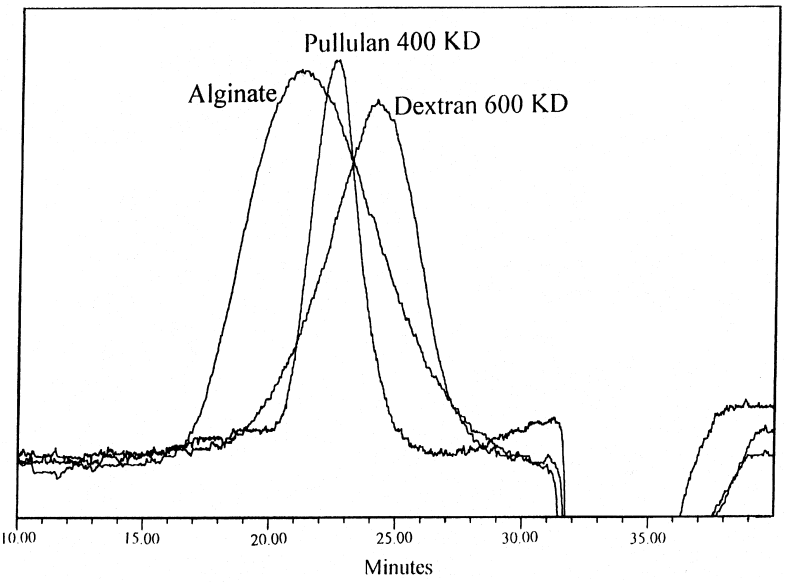

Fig. 10. Overlay of chromatograms of pullulan (400 000), dextran (600 000) and alginate with pH 4 buffer solution as mobile phase and RI detection, showing that SEC retention is strongly dependent on the chemical composition of sample molecules.

alginate would have a larger size and greater intrinsic viscosity than pullulan and dextran.

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