System Development for Size Exclusion Chromatography of Starch Hydrolysates

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Synopsis

A system for the aqueous size exclusion chromatography (SEC) of starch hydrolysates has been developed. Sepharose CL is the chromatographic support material, and aqueous sodium hydroxide solution is the eluent. Various factors affecting the resolution of the proposed system are discussed. Support materials having small particle size and narrow particle size distribution are necessary for maximizing separation efficiency. The ionic strength of the eluent, however, has a negligible effect on separation efficiency. The fractionation range of the system has been broadened by connecting in series columns containing two different Sepharose-CL gel types according to the bimodal pore size distribution concept. Well-characterized sodium polystyrene sulfonate and dextran standards are used to calibrate this SEC system. The Coll–Prusinowski calibration procedure leads to a linear calibration curve over a wide molecular weight domain.

INTRODUCTION

Size exclusion chromatography (SEC) is an important technique for determining the molecular weight and the molecular weight distribution of polymers in dilute solution.[1,2] The development of SEC for polymers soluble in organic solvents has been rapid. In contrast, the characterization of water-soluble polymers by SEC is not completely understood.[3,4] Some of the reasons for the less advanced state of aqueous SEC are: (i) difficulties in obtaining chromatographic supports for aqueous systems that possess the necessary separation characteristics; (ii) a lack of readily available, monodisperse water-soluble polymer standards; and (iii) an inadequate theoretical understanding of the factors governing separation in aqueous SEC.

In this paper, we report on the development and optimization of an aqueous SEC system for starch characterization. Effects of factors such as particle size of the chromatographic support, flow rate of the eluent, and solvent ionic strength on the observed separation efficiency are discussed. A method for increasing the separation range of the column is also demonstrated. Although the developments reported here were specifically undertaken for starch characterization, the concepts are equally valid for the analysis of many polymers of biological interest such as proteins, other polysaccharides, and nucleic acids.

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BACKGROUND

Column selection in SEC is based largely on the required molecular weight range of separation and the nature of the sample–solvent combinations. It has been shown in previous studies that an attractive SEC system for the study of starch liquefaction would employ crosslinked Sepharose gel supports with strong aqueous alkali as eluent.[5–8] The successful application of SEC to starch analysis requires that the system have a wide molecular weight fractionation range. No single type of Sepharose gel possesses the necessary separation capability for such studies. However, an SEC system which is useful for starches can be designed by joining in series columns containing different Sepharose gel types. The correlations for measuring the separation efficiency of chromatographic systems as well as some of the factors which affect separation are briefly discussed in the following paragraphs.

Measurement of Separation Efficiency

The separation efficiency of a set of SEC columns is expressed in terms of the number of theoretical plates per meter of the column set. The plate count N is experimentally determined using the following equation:

$$N = \frac{1600}{L}\left(\frac{V_R}{W}\right)^2 \tag{1}$$

where L is the column length (cm), W is the peak base width as determined from the intersections of the tangents drawn through the inflection points of the chromatographic trace with the baseline, and V_R is the peak elution volume of a solute whose molecular size is small enough for it to be able to permeate through the total internal pore volume of the column.[1] In this study, glucose was used as the solute for determining V_R. Alternately, the separation efficiency can be expressed in terms of plate height H, which is the reciprocal of the number of plates, N; i.e.,

$$H = N^{-1} \tag{2}$$

Effect of Eluent Flow Rate on Separation

The following relationship between plate height and eluent flow rate was proposed by van Deemter et al.[9]:

$$H = A + B/v + Cv \tag{3}$$

where A, B, C, are constants and v is the eluent linear velocity. The first term on the right-hand side of eq. (3) is associated with eddy diffusion, the second with axial diffusion, and the third with mass transfer effects. A graphical representation of the contributions of these three effects to plate height is shown in Figure 1. Since the axial diffusion term decreases with flow rate while the mass transfer term increases with it, there exists a nonzero value of flow rate at which the plate height is a minimum, i.e., separation occurs at maximum efficiency. For most separations, it is impractical to perform the separation at the optimum flow rate because of unacceptably long analysis times. Therefore, some separation efficiency is generally sacrificed for experimental convenience.

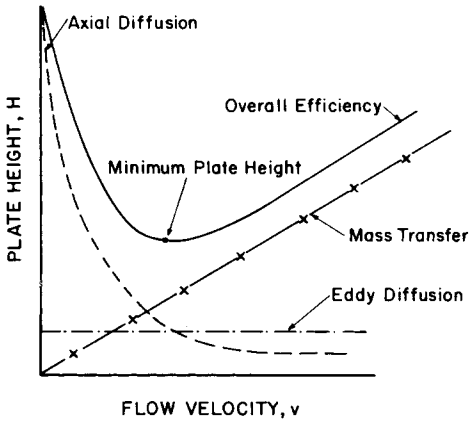

Fig. 1. Variation of SEC plate height with flow rate (Van Deemter eq.).

Effect of Particle-Size on Separation

Separation efficiency of a SEC system can be improved by modifying operating parameters other than eluent flowrate. The equation that has been proposed for expressing the relationship between plate height H and diameter d_p of the stationary chromatographic phase is[10]

$$H = C_{SL}\frac{vd_p^2}{D_{SL}} + \frac{1}{1/ad_p + D_M/C_M v d_p^2} \tag{4}$$

where C_{SL} is the constant associated with stationary liquid phase mass transfer, D_{SL} is the solute diffusion coefficient in stationary liquid phase, D_M is solute diffusion coefficient in mobile phase, and a,C_M are constants for a given SEC system. The smaller the particle size d_p, the smaller is the plate height H. Therefore, SEC separation efficiency can be increased by using smallest available particles as chromatographic support.

Ionic Strength Effects in Aqueous SEC

Many polymers which need to be characterized by aqueous SEC are polyelectrolytic in nature. The separation of such polymers by SEC depends upon the ionic strength of the eluent. In systems where the polymer and the support are oppositely charged, the polymer elutes later as the electrostatic attractions retard movement of the polyion through the porous matrix.[11] Additional ionic effects such as ion exclusion[12] and ion inclusion[13] are also likely to affect separation. Polyelectrolytes can be used as secondary standards in aqueous SEC for the study of other water-soluble polymers provided these polymer–solvent–support interactions are suppressed. In general, addition of simple salts to the eluting media overcome such interactions and thereby eliminate complications.[4]

EXPERIMENTAL

Two types of Sepharose gels, CL-6B and CL-2B (Pharmacia Fine Chemicals, Piscataway, N.J.), were used in these studies. Wet sieving was employed to fractionate the gel particles. The method involved slurrying 20–30 g of Sepha-

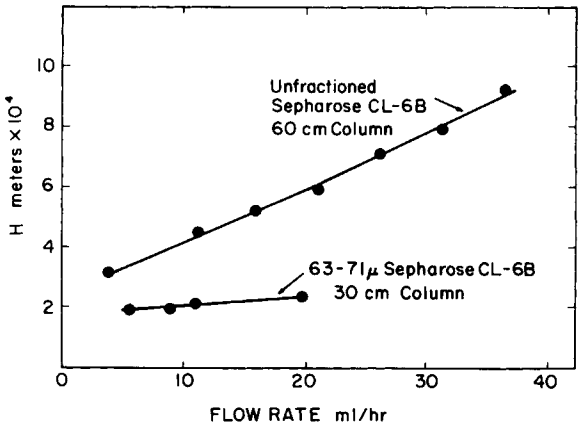

Fig. 2. Variation of plate height with flow rate for unfractionated and 63–71 μm Sepharose CL-6B gel columns.

rose gel in 500–700 mL of water and sieving the resultant slurry through a set of four 8-in. sieves (71 μm, 63 μm, 40 μm, and 25 μm openings). Water containing 1 drop/L Triton X-100 surfactant was sprayed constantly onto the top sieve, and the entire apparatus was vibrated. 2 L of water with surfactant percolated through the sieves. An additional 4–6 L of water without surfactant was subsequently sprayed onto the sieve shaker. This procedure was repeated with additional quantities of the gel until sufficient amounts of desired fractions had been collected to pack SEC columns. The fractionated gels were stored in sodium azide solutions to prevent microbial growth as recommended by the manufacturer. Prior to packing SEC columns with these materials, the gels were resuspended in appropriate solvents and degassed.

A detailed description of the SEC apparatus used in this study may be found elsewhere.[8] The detecting unit was a differential refractometer (Water Associates, Milford, Mass.). Differential refractive index responses are very sensitive to changes in temperature. To minimize systematic fluctuations in detector response due to temperature variations, the refractometer and chromatographic columns were thermostated at 25.0 ± 0.2°C with a circulating water bath. In addition, the refractometer, chromatographic columns, injector, and pulse dampener were placed in an insulated plexiglass box. The system designed in this fashion maintains a stable base line and is convenient to employ for routine polymer characterization.

The chromatographic columns were $\frac{3}{8}$-in. diameter, 304 stainless steel pipes of various lengths (as indicated below) capped with 2-μ end fittings (Waters Associates, Milford, Mass.). A sample size of 50 μL was used throughout these experiments. The sample concentration was 0.5% by weight in all cases. The

TABLE I
Properties of Sepharose-CL Gels

Gel type and particle size	Number of theoretical plates/m		
	Unfractionated	63–71 μ	40–63 μ
Sepharose CL-2B 60–250 μ	950	2900	
Sepharose CL-6B 40–210 μ	1900	4200	5300

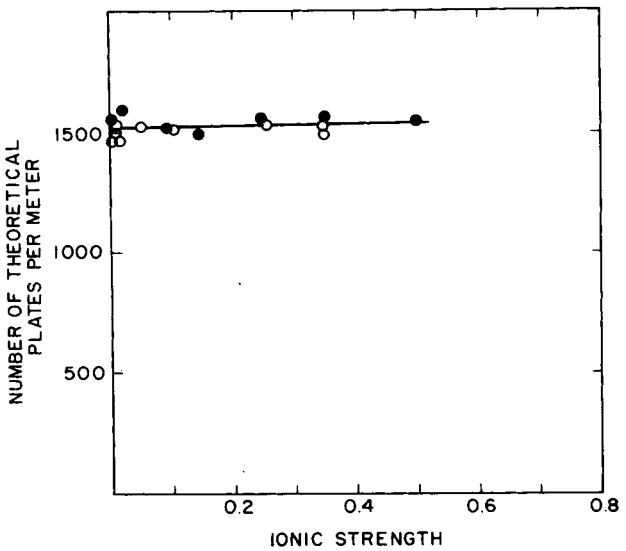

Fig. 3. Effect of ionic strength on number of theoretical plates for unfractionated Sepharose CL-6B gel column in NaOH (●) and NaCl (○) solutions. Column: 60 cm, flow rate: 12.1 mL/h.

chromatographic system was calibrated using two series of well-characterized polymers: sodium polystyrene sulfonates (NaPSS) obtained from Pressure Chemical Co. (Pittsburgh, Pa.) and dextrans obtained from Pharmacia Fine Chemicals (Piscataway, N.J.). The characteristics of these polymer standards were previously reported.[14]

Starch hydrolysates were prepared by hydrolyzing purified cornstarch (wet-milled process) with commercial grade α-amylase (Novo Labs, Wilton, Conn.) in aqueous solutions for various periods of time. The depolymerization was stopped and the SEC samples prepared by diluting the hydrolysate with an appropriate aqueous alkali to yield a 0.5% by weight starch solution. A detailed description of these procedures is given elsewhere.[15]

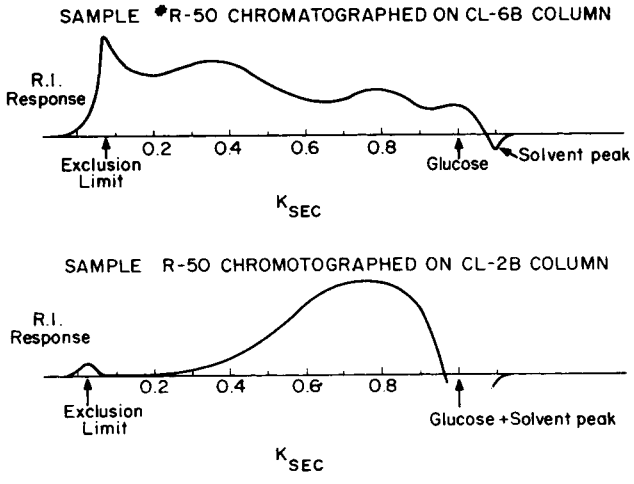

Fig. 4. Fractionation of starch sample R-50 on Sepharose CL-6B and Sepharose CL-2B column.

RESULTS AND DISCUSSIONS

Effects of Eluent Flow Rate and Support Particle Size on Separation

The partitioning of macromolecules in SEC between the stationary phase within the porous packings and the mobile eluent phase occurs solely by diffusion between the two phases.[1] The mobile phase velocity affects the solute diffusion coefficient. The efficiency of separation in SEC is, therefore, related to the eluent flowrate as represented by eq. (3). The experimentally determined relationships between the plate height and flow rate for a 60-cm unfractionated Sepharose CL-6B column and a 30-cm column containing the same gel in the particle size range of 63–71 μm are shown in Figure 2. The linearity of the relationship between plate height and flow rate for either column indicates that these SEC columns are operating in a mass transfer limited region. A small change in eluent flowrate will greatly affect the performance of the unfractionated Sepharose CL-6B column. Such dependency is minimal for Sepharose CL-6B that has been fractionated to a range of 63–71 μm. Thus, particle size distribution is an important parameter to be considered during the design of SEC columns.

The effects of gel type and particle size range on the number of theoretical plates per meter are shown in Table I for 30-cm-long SEC columns operating at a flow rate of 8.1 mL/h. Plate count increases with the degree of crosslinking of the gel. An unfractionated Sepharose CL-6B column has nearly twice the number of theoretical plates per meter as a similar Sepharose CL-2B column. For a given gel type, the chromatographic efficiency increases with decreasing particle size. Fractionation of either Sepharose CL-6B or CL-2B into smaller particle size and narrower particle size distribution results in a significant increase in the plate count. This observation is in agreement with the predictions of eq. (4). Design of an efficient SEC system thus requires support materials having small particle size and a narrow particle size distribution.

Effect of Eluent Ionic Strength on Separation

Ionic strength of the eluent is an important factor that affects separation in aqueous SEC.[4] This effect may be especially pronounced if ionic strength variations cause osmotic swelling of the Sepharose gel particles. The relationship between separation efficiency (measured in terms of the number of theoretical plates per meter) and eluent ionic strength was studied using a 60-cm-long unfractionated Sepharose CL-6B column. Various concentrations of NaOH and NaCl solutions were the eluents, and the flow rate through the column was maintained at 12.1 mL/h. The effect of ionic strength on the separation efficiency of this column was found to be minimal, as shown in Figure 3. Therefore, even though eluent ionic strength variations alter macromolecular conformation in solution[16] and thus affect SEC elution profile,[14] the efficiency of separation is unaffected by such ionic strength changes.

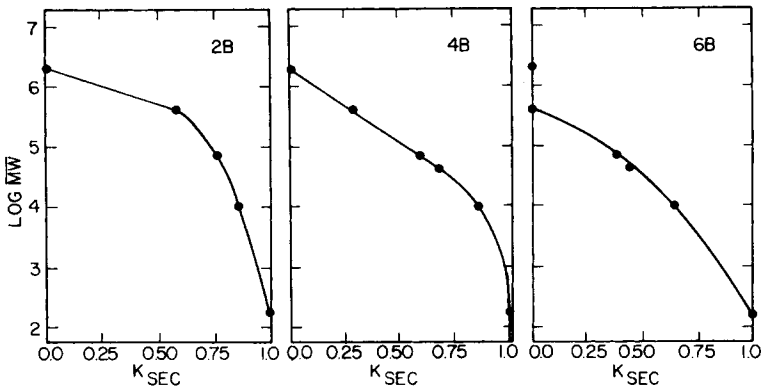

Fig. 5. Molecular weight fractionation range of dextran standards on various Sepharose gels.

Extension of Fractionation Range

One of the objectives of the chromatographic system being developed is to be able to follow the changes in the molecular weight distribution of starch as it is undergoing liquefaction. The desired fractionation range, therefore, encompasses molecular weights of 180 to $\sim 1.0 \times 10^8$.[17] No particular type of Sepharose-CL column is capable of fractionating molecules over such a wide molecular weight range. The problems encountered in trying to fractionate a starch sample on a single type of Sepharose-Cl column are evident from Figure 4. When this particular sample is chromatographed on a Sepharose Cl-6B column, ade-

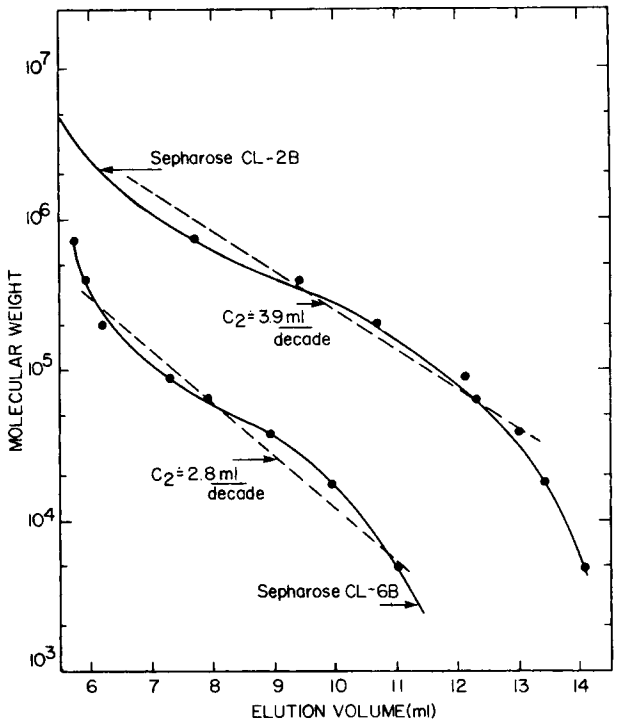

Fig. 6. Molecular weight calibration curves for NaPSS in 0.185 NaOH in Sepharose CL-2B and CL-6B supports.

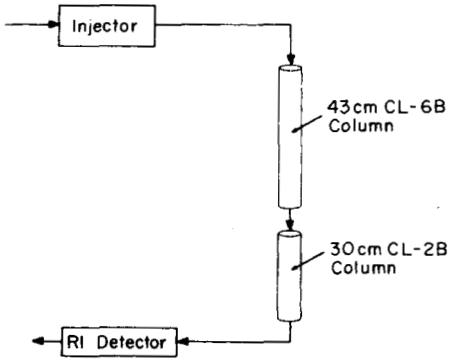

Fig. 7. Series column network.

quate resolution is obtained on the lower end of the molecular weight distribution—the portion of the sample possessing high molecular weights is unresolved. The opposite is true when the same sample is analyzed on a Sepharose CL-2B column—the information contained in the lower end of the molecular weight distribution is lost. Thus, there is a need to combine several types of Sepharose-CL columns in a SEC system, thereby increasing its fractionation range.

The traditional approach for extending the fractionation range of a SEC system has been to combine in series all the available columns which have separation capabilities in the molecular weight range of interest. However, this mode of SEC fractionation range extension has been shown not to be optimal, and most often leads to longer analysis times, nonlinear calibration curves, and lower column efficiency. Yau and co-workers[18] determined that the most efficient

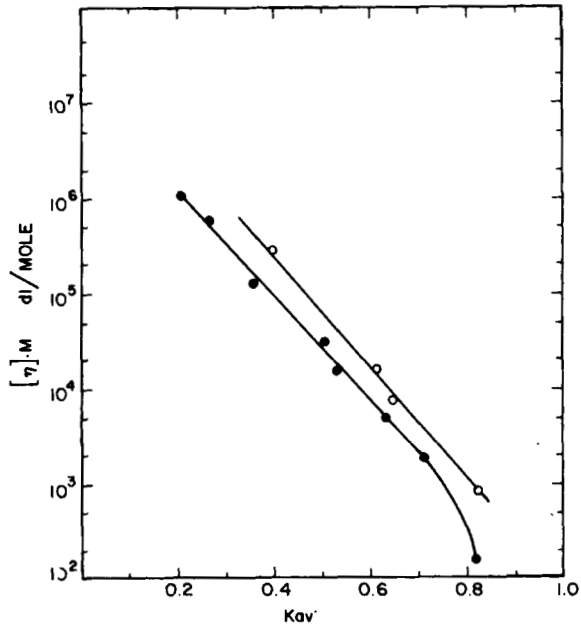

Fig. 8. Grubisic calibration of combined Sepharose-CL column. (○) Dextrans; (●) sodium polystyrene sulfonates.

SEC separation will result if narrow pore size distribution support particles are used and their molecular weight separation ranges are nonoverlapping. Maximal linearity of the calibration curve over an extended range is obtained when columns containing equivalent interstitial volumes and capacities are used.

Sepharose gels are not well characterized, and hence many of the parameters required to properly apply the bimodal pore size distribution concept[18] are unavailable. Pore size distribution for these materials cannot be obtained because their mechanical weakness preclude mercury porosimetry measurements. Another property of the gels necessary for the application of the proposed concept is their fractionation range. The approximate molecular weight range of separation of the three commercially available cross-linked Sepharose gels is shown in Figure 5. The fractionation ranges of all the gels overlap to some extent; however, Sepharose CL-6B and CL-2B seem to be the most compatible for extending the molecular weight fractionation range. Thus, these two gel types were characterized in order to design a SEC series network for starch liquefaction.

A linear calibration curve in SEC is desired to facilitate data handling. The attainment of this objective requires the determination of the calibration curves and the interstitial volumes of each of the gel types. The molecular weight calibration curves for 30-cm-long columns of Sepharose CL-6B and CL-2B are shown in Figure 6. Sodium polystyrene sulfonate (NaPSS) samples in 0.185 N NaOH were used as standards. The separation capacity of an individual column

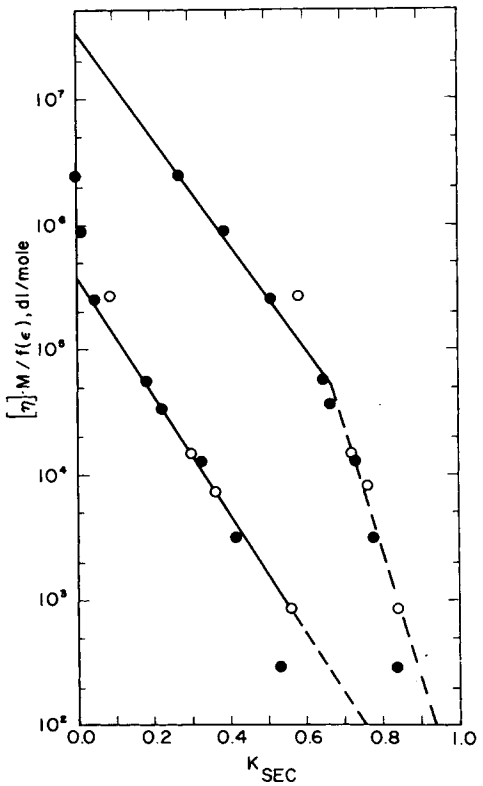

Fig. 9. Coll–Prusinowski plots for Sepharose CL-6B (lower curve) and CL-2B (upper curve) in 0.185 N NaOH. (○) Dextrans; (●) sodium polystyrene sulfonates. Both columns: 30 cm long; flow rate of eluent: 8.1 mL/h.

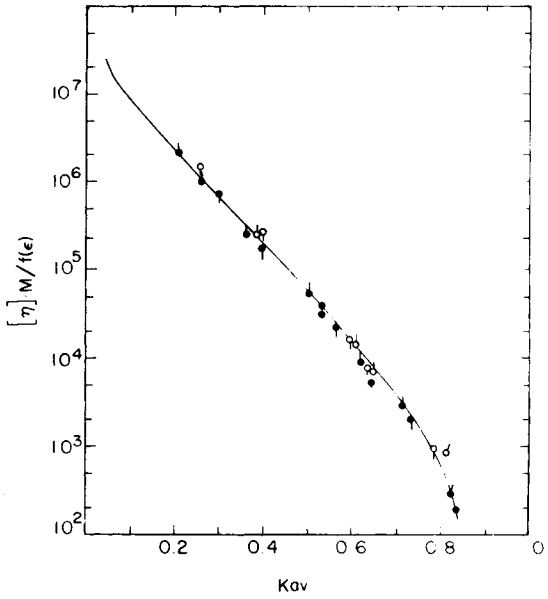

Fig. 10. Coll–Prusinowski plot of combined gel column in 0.185 N (downward pips) and 0.501 N (upward pips) NaOH. (○) Dextrans; (●) sodium polystyrene sulfonate.

is the slope of the elution volume vs. the logarithm of molecular weight curve.[18] Sepharose CL-6B has a capacity of 2.8 mL/decade while the value for Sepharose CL-2B is 3.9 mL/decade. The interstitial volumes of the two columns were also estimated by measuring the void volume of the column with a high molecular weight marker and the total mobile phase volume with glucose. The interstitial volumes for 30-cm-long Sepharose CL-6B and Sepharose CL-2B columns are 10.0 mL and 15.5 mL, respectively. The application of Yau's bimodal pore size distribution concept requires that the capacity and the interstitial volumes of the two columns connected in series be equal. To equate the capacity values for the Sepharose CL-6B and CL-2B columns, the ratio of column lengths for the CL-6B:Cl-2B supports must be 1.39 while, to equate interstitial volumes, a column length ratio of CL-6B:CL-2B = 1.5 must be employed. For the system reported in this paper, an intermediate value of 1.44 was chosen for the column length ratio. Thus, a 43.2-cm-long Sepharose CL-6B column was connected in series to a 30-cm-long Sepharose CL-2B column, as shown in Figure 7. Both columns were packed with the respective gel types having a particle size range of 63–71 μ.

Calibration of the SEC System

Calibration procedures based on molecular size in solution, as discussed in a previous paper,[14] were tested for the SEC system described in the preceding section. Two series of well-characterized polymers, NaPSS and dextrans, were used as standards. The eluent was 0.185 N NaOH. The results, according to the calibration procedure of Grubisic et al.,[19] are shown in Figure 8. A common calibration curve is not obtained for the two sets of standards. The polyelectrolyte elutes earlier than the dextran having the same $[\eta]$·M value. Thus,

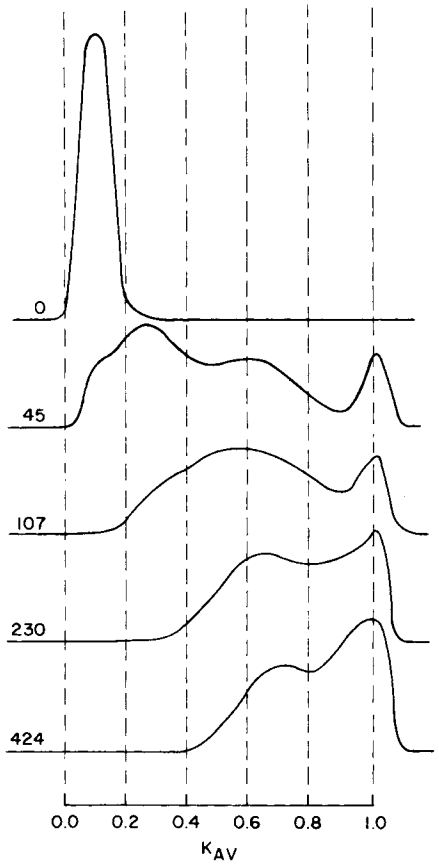

Fig. 11. Sample starch chromatograms on series column network. Numerals on curves indicate hydrolysis time (s).

Grubisic's calibration method is inapplicable to the SEC system under investigation.

It has been reported that the Coll–Prusinowski calibration procedure[20] is useful for the calibration of unfractioned Sepharose CL-6B columns.[14] We tested its validity for fractionated Sepharose CL-6B and CL-2B columns as well as a series network of the two (constructed in accordance with the bimodal pore size distribution concept). The results of the calibration procedure for the individual columns used in the series network are shown in Figure 9. A common calibration curve is obtained for both the NaPSS and dextrans. The linear calibration range for Sepharose CL-6B in units of hydrodynamic volume (dL/mol) extends from approximately 3×10^5 to below 1×10^3. The equivalent fractionation range for Sepharose CL-2B is approximately 5×10^4 to about 3×10^7. There is some overlap in the linear fractionation ranges of the sepharose CL-6B and CL-2B columns. Hence, the two are not ideally suited for connecting in series according to the bimodal pore size distribution concept. In spite of this drawback, the network of two columns exhibits calibration linearity in the range of 5×10^2–2×10^7 hydrodynamic volume units, as shown in Figure 10. The eluents used were 0.185 N and 0.501 N NaOH. In this range of eluent ionic strength, both the NaPSS and dextrans produced a common calibration curve. The efficiency of

this column network was determined to be 3300 theoretical plates/m, which is a value intermediate between those of the individual columns comprising the network.

The SEC column network was employed to determine molecular weight distribution changes during starch liquefaction. The detailed description of the exact reaction conditions used are found elsewhere.[15] Figure 11 is representative of the type of molecular information obtainable with this combined gel column. The numbers associated with each of the chromatograms indicate hydrolysis time in seconds. Four distinct chromatographic peaks are observed: one at the exclusion limit of the column, one at the low molecular weight limit of resolution, and two at intermediate molecular weight ranges. In contrast to published reports, liquefaction does not seem to result in Gaussian molecular weight distribution for the hydrolyzed starches.[5–7] This observation clearly indicates the benefits of using aqueous SEC in describing the dynamics of starch liquefaction.

CONCLUSION

The methodology for the development of an aqueous SEC system for the analysis of starches and other water-soluble polymers is discussed and demonstrated. Chromatographic support materials having small particle size and narrow particle size distribution are necessary for maximizing the separation efficiency. The ionic strength of the eluent, however, has a negligible effect on separation efficiency. The fractionation range of the SEC system can be widened by connecting in series two columns of different packing types according to the bimodal pore size distribution concept. The Coll–Prusinowski calibration procedure is applicable to the resultant column network. Finally, the utility of this method of aqueous SEC for characterizing starch digests is demonstrated.

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