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MOLECULAR-SIEVE CHROMATOGRAPHY OF AMYLOSE AND DEXTRAN OVER POROUS GLASS*

F. R. DINTZIS AND R. TOBIN

*Northern Regional Research Laboratory***, *Peoria, Ill. 61604 (U.S.A.)*

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SUMMARY

Porous glass of 370 Å average pore diameter is useful for fractionating amylose and dextran of weight-average molecular weights ranging from 2×10^5 to 2×10^4 and at flow-rates of 2.4–2.9 ml/h,cm^2 at room temperatures. Amylose and dextran in methyl sulfoxide–water (95:5) or 4 *M* guanidine hydrochloride as solvents exhibit the same calibration curve; hence, commercially available dextran fractions may serve to calibrate the column for use with amylose. Polystyrene fractions in tetrahydrofuran exhibit a different calibration curve. Thus the universal calibration concept does not apply to all three polymers in our column.

INTRODUCTION

Molecular-sieve chromatography is useful in many areas of molecular separations[1]. Barker *et al.*[2] have applied chromatography over porous glass beads to the fractionation of dextran and hyaluronic acid. Macromolecular fractionations are now routinely carried out with a variety of porous media and suitable solvents.

One purpose of our study was to determine whether or not the "universal calibration concept" is valid and applicable to amylose. This concept states that polymer species with the same value of the product $\overline{M}_w\times[\eta]$ should elute at the same V_e from the same chromatographic column, where $[\eta]$ is the intrinsic viscosity, $\overline{M}_w$ is the weight-average molecular weight as determined by light scattering, and V_e is the elution volume[3]. Another purpose of this paper was to present results of our fractionating amylose over a column of porous glass beads and to show that the column is a good analytical tool for molecular weight determinations of amylose.

Methyl sulfoxide–water (95:5) is an economical and satisfactory solvent for permeation chromatography over porous glass. Although expensive, 4 *M* guanidine hydrochloride (GHCl) is successful as a solvent for amylose fractionation. Amylose is stable in methyl sulfoxide for long periods. At room temperatures, amylose is stable in 4 *M* GHCl for at least two weeks. In aqueous solutions or dilute salt solutions, amylose obviously tends to retrograde or become insoluble at concentrations of about 0.1% or greater. This retrogradation is especially severe with maize amyloses, which have no

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phosphate groups. The two solvents selected are ones in which maize amylose is eluted through our column without retrogradation or adsorption onto the glass.

MATERIALS AND METHODS*

Polystyrene samples of narrow molecular-weight distributions (MWD) were purchased from Waters Ass. (Milford, Mass., U.S.A.) and dextran fractions from Pharmacia (Piscataway, N.J., U.S.A.). Amylose fractions, prepared at the Northern Laboratory from dent maize by fractional precipitation with ethanol from methyl sulfoxide solution, were stored in 95% methyl sulfoxide[4]. Molecular weights of these fractions were determined by light scattering in 4 *M* GHCl in a modified Brice-Pheonix instrument (Pheonix Instrument Co., Philadelphia, Pa., U.S.A.). Solution viscosities were measured in Cannon-Manning (State College, Pa., U.S.A.) semimicro viscometers.

GHCl of 98% purity was purchased from Eastman-Kodak (Rochester, N.Y., U.S.A.) and used without further purification. A sufficiently large batch of 4 *M* GHCl of refractive index $\bar{n}=1.401$, was prepared for both light scattering and column elution purposes. The variability in composition of commercially available GHCl makes this precaution necessary[5]. Fisher (Pittsburgh, Pa., U.S.A.) certified reagent-grade tetrahydrofuran (THF) was used with the polystyrene fractions. Drum lot methyl sulfoxide from Crown Zellerbach (Camas, Wash., U.S.A.) was redistilled under vacuum. This purification step removed a yellow-brown substance that otherwise would adsorb onto the glass beads and eventually alter column characteristics. Methyl sulfoxide solvent is adjusted to a refractive index of approx. 1.471, which corresponds to about methyl sulfoxide–water (95:5).

A standard Pharmacia column of 2.5-cm diameter, 100-cm length, was packed with porous glass to a 92-cm bed length. The packing consisted of Corning (Corning, N.Y., U.S.A.) porous glass, CPG-10-370, lot No. 82618, of 370 Å average pore diameter and a particle size of 75–125 μm. All fittings were solvent resistant, and the column was jacketed and kept at 25.0°. Reverse flow was used in all runs, and samples of up to 1.0 ml were injected into the bottom of the column. Eluted peaks initially were measured in collected fractions by a phenol–sulfuric acid–total carbohydrate determination modified for a Technicon AutoAnalyzer[6] (Technicon, Tarrytown, N.Y., U.S.A.). The specific rotations of amylose, dextran and maltose are high enough that column effluent in later runs was monitored with a Bendix (Nottingham, Great Britain) automatic polarimeter having a flow cell of 5.0-cm length. Maltose served as a marker to indicate liquid volume of the packed column. Eluted peaks of polystyrene in THF were measured at 254 nm with an LKB Uvicord II detector (LKB, Rockville, Md., U.S.A.).

Aliquots of amylose fractions in 4 *M* GHCl were obtained as needed by precipitating suitable amounts of amylose from the stock solutions with ethanol. The alcohol-damp precipitate was then dissolved in 4 *M* GHCl and dialyzed against 4 *M* GHCl in a regenerated cellulose dialysis tubing (Union Carbide, Chicago, Ill., U.S.A.). Concentrations were determined by optical rotation. Specific rotation at the sodium D line, $[\alpha]_D$, for clinical dextran is 205 in 4 *M* GHCl and 212 in methyl sulfoxide–water

* Mention of firm names or trade products does not imply that they are endorsed or recommended by the Department of Agriculture over other firms or similar products not mentioned.

(95:5). Our T-10 dextran has sufficiently low $\overline{M}_w$ material that for this sample $[\alpha]_D$ is 198.7 in 4 *M* GHCl and 208.5 in 95% methyl sulfoxide. For amylose, $[\alpha]_D$ is 203 in 4 *M* GHCl and 189 in 95% methyl sulfoxide.

The polymers used are relatively linear. Polystyrene is produced by anionic polymerization with little or no branching. Amylose from natural sources is essentially a linear polymer with a small percentage of long-chain branches[7]. The backbone is linked α (1→4) with side chains thought to be linked mainly at the 6 position. The microorganism *Leuconostoc mesenteroides* B-512 produces dextran[8]. These polymers are α-D-glucans with mostly (1→6) backbone links and side chains linked at the 3 position. There are approximately 5% branching points wherein about 40% of the side chains consist of one D-glucose unit, at least 45% are two D-glucose units long, and the remaining 10–15% of side chains are greater in length[9]. Polystyrene standards are of low polydispersity with $\overline{M}_w/\overline{M}_n < 1.1$. Dextran calibration samples have $\overline{M}_w/\overline{M}_n$

TABLE I

CHARACTERISTICS OF POLYMER FRACTIONS

Polymer		$\overline{M}_w \times 10^{-5}$	$\overline{M}_n \times 10^{-5}$	$[\eta]$(ml/g)	$(\overline{M}_w \times [\eta]) \times 10^{-6}$	V_e(ml)
Polystyrene in THF standard No.						
61970		21.45	17.80	381	818	203
25166		4.11	3.92	120	49.3	204
41984		1.73	1.64	65.4	11.3	234
41995		0.982	0.962	44.0	4.3	263
25170		0.51	0.49	27.8	1.42	290
25168		0.1985	0.1965	14.4	0.286	325
25171		0.103	0.097	9.08	0.0935	340
Dextran in 95% methyl sulfoxide						
Designation	Lot No.					
T-500	3207	5.16	2.12	78.0	40.2	205
C_1	–	2.20	–	60.0	13.2	228
T-150	921	1.54	0.860	52.5	8.08	253
T-70	693	0.695	0.395	35.6	2.47	293±2.5
T-20	7968	0.223	0.150	20.9	0.466	326
T-10	3205	0.093	0.057	13.3	0.124	351
Dextran in 4 M GHCl						
T-70	693	0.695	0.395	26.5	1.84	302
T-10	3205	0.093	0.057	10.0	0.093	357
Amylose in 95% methyl sulfoxide						
Fraction designation						
B		4.60		103.5	47.6	206
C		3.10		86.5	26.8	205
VII		2.20		73.0	16.0	215±4
E		2.05		70.0	14.3	230
F		1.16		46.0	5.32	266±2
G		0.23		19.5	0.45	329
H		0.11		13.5	0.15	348±3
Amylose in 4 M GHCl						
F_1		1.16		42.5	4.93	272±3
Maltose in 95% methyl sulfoxide						387

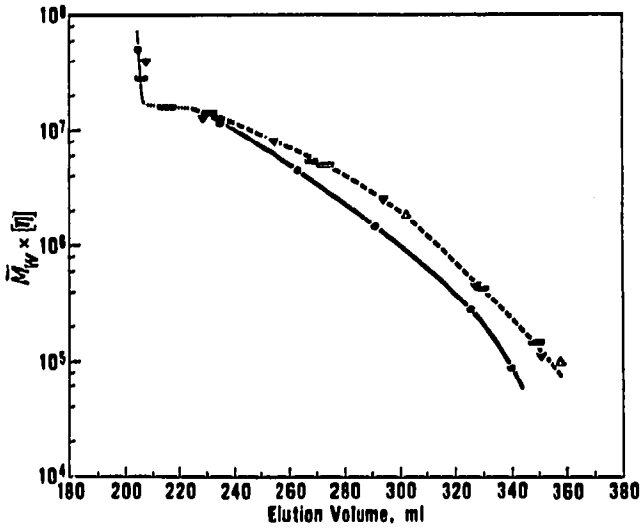

Fig. 1. Elution characteristics of polymers in CPG-10-370 column. ●, polystyrene in tetrahydrofuran; ▼, B-512 dextran in methyl sulfoxide–water (95:5); △, B-512 dextran in 4 *M* GHCl; ■, dent maize amylose in methyl sulfoxide–water (95:5); □, dent maize amylose in 4 *M* GHCl.

values of 1.5 to 2.0, and our laboratory-prepared amylose fractions are expected to have the same order of polydispersities.

RESULTS

Table I summarizes experimental measurements and characteristics of the polymer fractions. Number-average molecular weights, $\bar{M}_n$, were not determined for our laboratory-prepared samples of dent maize amylose and the B-512 dextran sample, C_1. Intrinsic viscosities of linear polystyrene fractions in THF were calculated from the relation $[\eta] = 1.41 \times 10^{-2} M^{0.70}$, which has been shown to be valid for polymer $\bar{M}_w \geqslant 10^4$ (ref. 10).

The product $\bar{M}_w \times [\eta]$ *vs.* V_e, as determined in our column, for the various fractions in different solvents is plotted in Fig. 1. The universal calibration concept suggested by Benoit *et al.*[3] is valid for the two polysaccharides, amylose and B-512 dextran, in both 95% methyl sulfoxide and 4 *M* GHCl. Polystyrene in THF has a different behavior, as indicated by its distinctly separate $\bar{M}_w \times [\eta]$ *vs.* V_e plot.

Flow-rates of approximately 12–14 ml/h were used in our column without shifting peak position or distorting peak shape at sample concentrations of 4×10^{-3} g or less and a volume of 1.0 ml or less. Fractions in 95% methyl sulfoxide yielded altered curves and shifted elution volumes at flow-rates greater than 25–35 ml/h. Injected samples containing about 30×10^{-3} g or more solute in a sample volume of 1 ml or less yielded distorted elution curves.

One convenient measure of column efficiency is a reduced dispersion, *S*, which may be defined as the width of an eluted peak at its half height divided by V_e, though a usual definition of *S* is the standard deviation of the peak divided by V_e (ref. 11). While our eluted peaks are not gaussian, they are sufficiently symmetrical for the

TABLE II

REDUCED DISPERSIONS S*

Material	$\bar{M}_w \times 10^{-4}$	*S*	*Solvent*
Amylose	20.5	0.27	methyl sulfoxide–water (95:5)
	2.3	0.12	
Dextran	15.4	0.32	methyl sulfoxide–water (95:5)
	6.9	0.26	
	2.2	0.15	
Maltose	—	0.11	methyl sulfoxide–water (95:5)
Polystyrene	17.3	0.13	THF
	2.0	0.14	

* S= Width of eluted peak at half height/V_e.

parameter S to yield a useful approximation of efficiency. A comparison of S values measured in our column for various fractions is presented in Table II.

One of the uses of gel permeation chromatography (GPC) data is to calculate and compare MWD of different samples. The curve Pharmacia obtained by "gel filtration" for their T-70 dextran sample in water is reproduced in Fig. 2. Results on our sample of T-70 dextran eluted through our column in 95% methyl sulfoxide show reasonable agreement with the Pharmacia data, though obviously we did not obtain an identical MWD curve.

The possibility of amylose in methyl sulfoxide adsorbing onto glass beads was investigated by two methods. After the column calibration had been rechecked with several amylose samples, the peak area under a fraction G amylose elution curve was measured and the amount of eluted amylose was calculated. The calculated mass was within experimental error of the amylose contained in the injected sample. The second method consisted of stirring overnight a 50-ml sample of cleaned glass beads in 100 ml of 95% methyl sulfoxide containing 1 ml of 0.6% fraction F amylose. The solution was then decanted and the beads were washed five times with 100 ml of 95% methyl sulfoxide each time. Water (100 ml) and conc. H_2SO_4 (5 ml) were

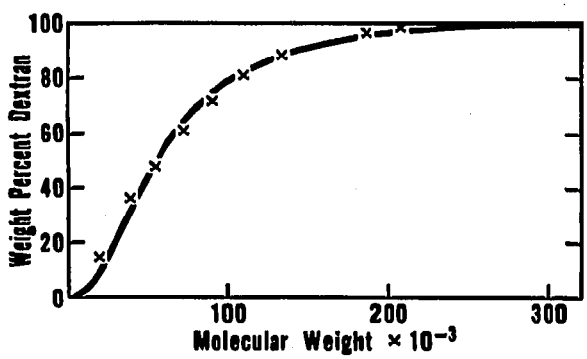

Fig. 2. Distribution curve of T-70 dextran. —, Results from gel filtration in water. Data provided by Pharmacia (appropriate distribution function supplied with each dextran fraction). ×, Results calculated from elution in 95% methyl sulfoxide over CPG-10-370 porous glass column.

added to the methyl sulfoxide-damp beads. This mixture was stirred and heated on a steam-bath for 1 h to hydrolyze any adsorbed amylose. The acid solution was then decanted and tested for carbohydrate by a phenol–sulfuric acid–total carbohydrate method[12]. No carbohydrate was detected. Apparently in a 95% methyl sulfoxide solution, amylose is not strongly adsorbed onto porous glass beads.

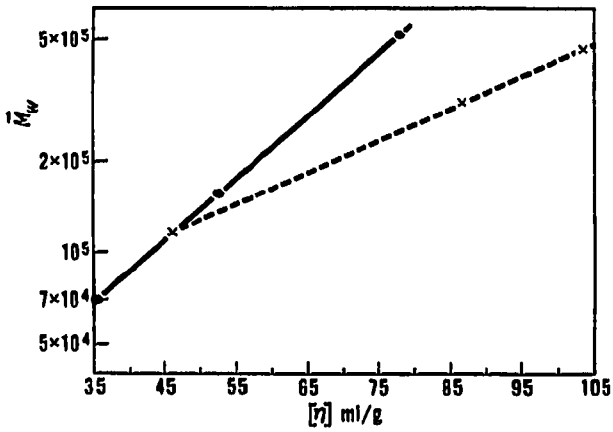

Fig. 3. Intrinsic viscosity–molecular weight behavior of (●) B-512 dextran and (×) dent maize amylose in 95% methyl sulfoxide.

Intrinsic viscosity–molecular weight relationships for the higher $\overline{M}_w$ fractions of dextran and amylose in 95% methyl sulfoxide are plotted in Fig. 3. These plots are linear within the $\overline{M}_w$ ranges indicated. Mark–Houwink equations obtained from these data are:

For B-512 dextran $$[\eta]=0.14\,\overline{M}_w^{0.39} \quad (1)$$

For dent maize amylose $$[\eta]=0.57\times 10^{-2}\,\overline{M}_w^{0.59} \quad (2)$$

Exponent values in these equations indicate that methyl sulfoxide is a better solvent for amylose than for B-512 dextran. Banks and Greenwood[13] found the following equation for potato amylose in methyl sulfoxide–water (99.8:0.2):

$$[\eta]=1.51\times 10^{-2}\,\overline{M}_w^{0.70} \quad (3)$$

The lower value of the exponent in eqn. 2 shows that additional water used in our solvent decreases its solvation for amylose compared to that of almost pure methyl sulfoxide. The exponent value of 0.39 (eqn. 1) for B-512 dextran is comparable to values obtained in water by Senti and coworkers[14], who calculated a value of 0.5 for fractions of $\overline{M}_w < 10^5$ and lower values for fractions of $\overline{M}_w > 10^5$.

DISCUSSION

As indicated by the two functions displayed in Fig. 1, the universal calibration concept is not valid in our column for all three polymers tested. The data presented in Table I are uncorrected for such factors as polydispersity of sample and axial disper-

sion of material through the column. We consider the two separate calibration functions to be a real effect that is outside of experimental error. Reasons for the difference in fractionation behavior between polystyrene and the two polysaccharides are not known.

For a given value of the product $\overline{M}_w \times [\eta]$ polysaccharide elutes at a larger V_e (Fig. 1). An interaction between glass surfaces and the polysaccharides that results in retardation of polysaccharide flow could explain the observed calibration differences. This explanation would require that the retardation be equivalent for both amylose and dextran in both 4 *M* GHCl and 95% methyl sulfoxide. In water solutions, amylose is strongly adsorbed onto the porous glass, whereas dextran is not. Thus adsorbtion forces between glass surfaces and the two polysaccharides in water differ greatly. These factors lead us to believe that attraction forces between porous glass surfaces and the polysaccharides may not explain differences between the calibration functions of polystyrene and the polysaccharides. However, Kennedy[15] points out that attraction forces between porous glass surfaces and proteins still operate in ionic aqueous media. Therefore, we believe one should not discard the possibility of adsorption forces retarding polysaccharide flow, for reversible adsorption could lengthen residence time of polysaccharide in the column.

Effects of polydispersity in GPC calibration are considered in a paper by Whitehouse[16]. He demonstrates that the effects of polydispersity upon $[\eta]$ may be significant. For two polymers of the same type and $\overline{M}_w$, with polydispersities of 1.1 and about 2, we estimate from the Whitehouse calculation that $[\eta]$ would decrease about 5% for the more polydisperse material. Therefore, a polydispersity correction applied to polystyrene standards used here would separate the polystyrene calibration curve farther from the polysaccharide calibration curve. The low polydispersity of polystyrene standards does not explain differences in permeation behavior between polystyrene and the polysaccharides.

An obvious possibility is that behavior of the two polysaccharides in 4 *M* GHCl and in 95% methyl sulfoxide is sufficiently different from that of polystyrene in THF that the equivalent hydrodynamic volume approximation is not valid for all three polymers. It is not known whether the failure to obtain a universal calibration curve is caused by an invalid equivalent hydrodynamic volume approximation or perhaps by differences in fractionation mechanisms during flow through porous glass. Failure to obtain a universal calibration curve is demonstrated also between polystyrene and polypropylene glycol[17].

Effects of column and flow factors that cause peak spreading are demonstrated in Table II, where the best *S* value obtained is 0.11 for the monodisperse "monomer", maltose. The sharp polystyrene fractions have *S* values that approach that of maltose as do two of the lower-molecular-weight polysaccharide fractions. Higher *S* values for the remaining polysaccharide fractions probably reflect higher polydispersities of these fractions. Values of *S* calculated from our data compare well with an *S* value of 0.22 estimated from data reported by Whelan[18] for the low-molecular-weight form (*i.e.*, about 45000) of an enzyme, pullulanase, which was refractionated on a Sephadex G-200 column of dimensions 2.54×88 cm. Thus, porous glass columns can provide efficiencies comparable to those obtained from porous gels.

A rigorous procedure for reducing GPC data to differential MWD curves is discussed and demonstrated by Yau and Fleming[19]. They emphasize that accurate

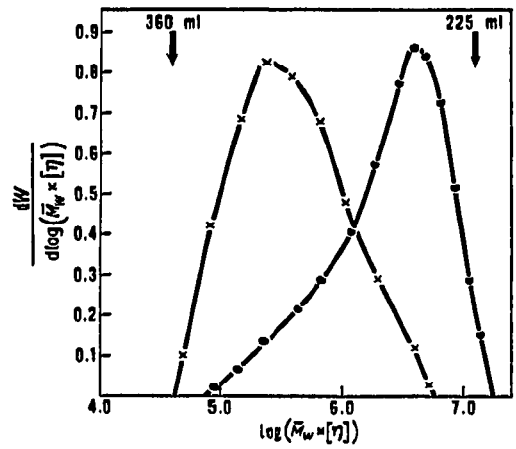

Fig. 4. Effective hydrodynamic volume distribution of (●) fraction T-70 B-512 dextran and (×) fraction G dent maize amylose in 95% methyl sulfoxide and 4 *M* GHCl. Arrows correspond to indicated elution volumes.

comparisons of GPC data, even when obtained on the same column, are generally possible only with consideration of differential calibration curves. One may use the function, log ($\bar{M}_w \times [\eta]$), in these calculations in place of the usual, log $\bar{M}_w$, to express results in terms of the universal calibration concept of equivalent hydrodynamic volumes. Fig. 4 displays the differential intrinsic viscosity–weight-average MWD curves of T-70 dextran and fraction G dent maize amylose in 4 *M* GHCl and 95% methyl sulfoxide. If assumptions contained in the universal calibration concept are valid for these solvent and polymer systems, then the curves represent distributions proportional to the effective hydrodynamic volumes of the two samples in either solvent.

Practical difficulties of using molecular-sieve chromatography to obtain accurate MWD of similar polymers are well known and documented. (For a short review and list of references, see ref. 20. See also refs. 17 and 21.) Data presented here show commercially available dextran samples may be used to calibrate the column for fractionation of amylose. Application of this system requires knowledge of $[\eta]$ of fractions of similar polydispersity before an $\bar{M}_w$ may be estimated. Fig. 1 shows the polysaccharide calibration curve is not linear over the entire fractionation range. Fig. 3 shows the $\bar{M}_w$ *vs.* $[\eta]$ relationship for the two polysaccharides is significantly different at high $\bar{M}_w$ values. A plot of $\bar{M}_w$ *vs.* $[\eta]$ (obtainable from Table I) for lower-molecular-weight fractions indicates nonlinearity below $\bar{M}_w = 70000$. These data show that use of proposed transformation equations of calibration curves which involve Mark–Houwink parameters are not practical over the entire fractionation range of our column[17,21].

Work presented here has been directed towards demonstrating the feasibility of applying molecular-sieve chromatography for the fractionation and molecular characterization of amylose. It is reasonable to expect that efforts to optimize column dimensions would result in either more efficient fractionation, more convenient elution time, or both of these. The availability of porous glass beads in a variety of

pore sizes allows an investigator to apply this permeation chromatography system to a wide range of amylose molecular weights. Advantages of porous glass over other permeation media have been discussed by Barker *et al.*[2]; some disadvantages have been described by Kennedy[15].

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