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Note

New high-performance gel permeation chromatographic system for the determination of low-molecular-weight amyloses

W. PRAZNIK* and R. H. F. BECK
Universität für Bodenkultur, Institut für Chemie, Arbeitsgruppe für Lebensmittel-, Umwelt- und Naturstoffchemie, Gregor Mendelstrasse 33, A-1180 Vienna (Austria)
and
W.-D. EIGNER
Universität Graz, Institut für Physikalische Chemie, Heinrichstrasse 28, A-8010 Graz (Austria)
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For the molecular characterization of native and hydrolysed amyloses and starches of various origins, low-pressure gel permeation chromatography (GPC) has frequently been used[1]. Recently high-performance gel permeation chromatographic (HPGPC) systems for the analysis of amyloses and starches have been discussed by several authors[2–8]. At first, porous glass, analogous to that used in low-pressure controlled-pore-size glass columns, was used for HPGPC[2]. With the development of high-pressure-resistant synthetic gels, however, the possibilities for the determination of amyloses and starches were extended. Both aqueous[2,5,6] or organic[3,4,7] eluents have been used, and dimethyl sulphoxide (DMSO) mixtures have been particularly popular.

For an exact determination of the molecular distribution of amyloses and starches it is necessary to calibrate the chromatographic system with polysaccharides of known molecular structures that are at least very similar if not identical. As Kuge *et al.*[6] have shown, calibration with dextrans is also possible, but the different intrinsic viscosities $[\eta]$ of the two different polymer series must be taken into consideration. The calibration can only be done by relating the elution volume to the hydrodynamic volume, $[\eta] \cdot MW$, of the polysaccharides.

A direct calibration is possible only with defined standards of the same or of a related polymer series. We took molecularly defined synthetic amyloses[9,10] and the commercially available structurally related pullulans as calibration standards. Their narrow distribution allows an exact calibration graph to be constructed that is especially suitable for low-molecular-weight amyloses, either native or degraded, but of course not for amylopectin.

This paper describes a new HPGPC system that uses an agarose column (Superose 6) that allows the molecular distribution of native amyloses and of hydrolysed starches to be determined.

MATERIALS AND METHODS

The hydrolytic degraded amyloses were prepared according to the method of

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TABLE I

MOLECULAR WEIGHT AND DISPERSITY FACTOR ($\overline{MW}_w/\overline{MW}_n$) OF CALIBRATION STANDARDS AS SHOWN IN FIG. 1

Synth. amylose	$\overline{MW}_w \cdot 10^{-4}$	$\overline{MW}_w/\overline{MW}_n$*	*Pullulan*	$MW \cdot 10^{-4}$	$\overline{MW}_w/\overline{MW}_n$
A-900	94.7	1.09	P-800	85.3	1.14
A-350	36.8	1.08	P-200	18.6	1.13
A-130	13.4	1.01	P-50	4.8	1.09
A-17**	0.24	1.13	P-5	0.58	1.07

* Determined by HPGPC distribution.
** Commercial product (Hayashibara).

Praznik *et al.*[11]. Commercial potato amylose (Avebe, Netherlands) was first purified by precipitation twice with butanol[12] to remove impurities. Hydrolysis was done in 0.1 *N* hydrochloric acid in 98% acetone at 38°C. The suspension was neutralized with 1 *N* sodium acetate after the desired reaction time. The precipitate was washed with distilled water and after that with acetone.

Amylose calibration standards were synthesized enzymically with potato phosphorylase[13]. The molecular weights of these amylose samples were determined by means of static low-angle laser light scattering. This method yields the weight-average molecular weight ($\overline{MW}_w$), and hence ensures a column calibration exactly to amylose. The molecular weight distributions thus determined turn out to be more accurate than those obtained from a GPC column calibrated just with dextran standards.

Low-molecular-weight amylose (Hayashibara, Japan) was characterized by its chemical reducing power and by low-pressure GPC on Biogel P-6[14]. Pullulan calibration standards were purchased from Shodex (Macherey-Nagel). The molecular parameters of these standards are given in Table I. Glucose and deuterium oxide were from Merck.

Amylose samples (10–20 mg) dissolved in 1 ml of 90% DMSO (Merck) at 60°C within 4 h. The clear or slightly opalescent solution was mixed with at least four volumes of methanol. The precipitate formed was centrifuged (10 min, 2000 *g*) and dissolved in 1 ml of water and bubbling steam to remove methanol.

The freshly prepared methanol precipitate of amylose seems to be in an amorphous state and is therefore readily soluble in water, as are cold water soluble and insoluble inulin precipitates[15]. The resulting aqueous solutions show retrogradation after *ca.* 1 h. The sample injection must therefore be done immediately after dissolving in water.

Pullulan standards are soluble directly in distilled water.

Low-angle light-scattering experiments were performed with a Chromatix KMX-6 (LDC/Milton Roy)[16,17]. The scattering angle was 4.8°. The amylose solutions were filtered (0.2 μm or 0.45 μm, cellulose acetate; Sartorius) directly into the sample cell. The scattering intensity was monitored with a strip chart recorder. According to light-scattering theory[18,19], this signal bears the information about the molecular weight of the investigated sample. At least four different sample concentrations were measured to carry out the extrapolation, and thus to determine the molecular weight ($\overline{MW}_w$).

A 50-μl volume of sample solution was applied with a Rheodyne 7125 loop valve to a Superose 6 column (Pharmacia) (300 × 10 mm I.D.); deaerated distilled water was used as solvent (LKB 2150 pump). A Waters RI 401 detector was connected with an LKB 2210 flat-bed recorder. The separation was performed at room

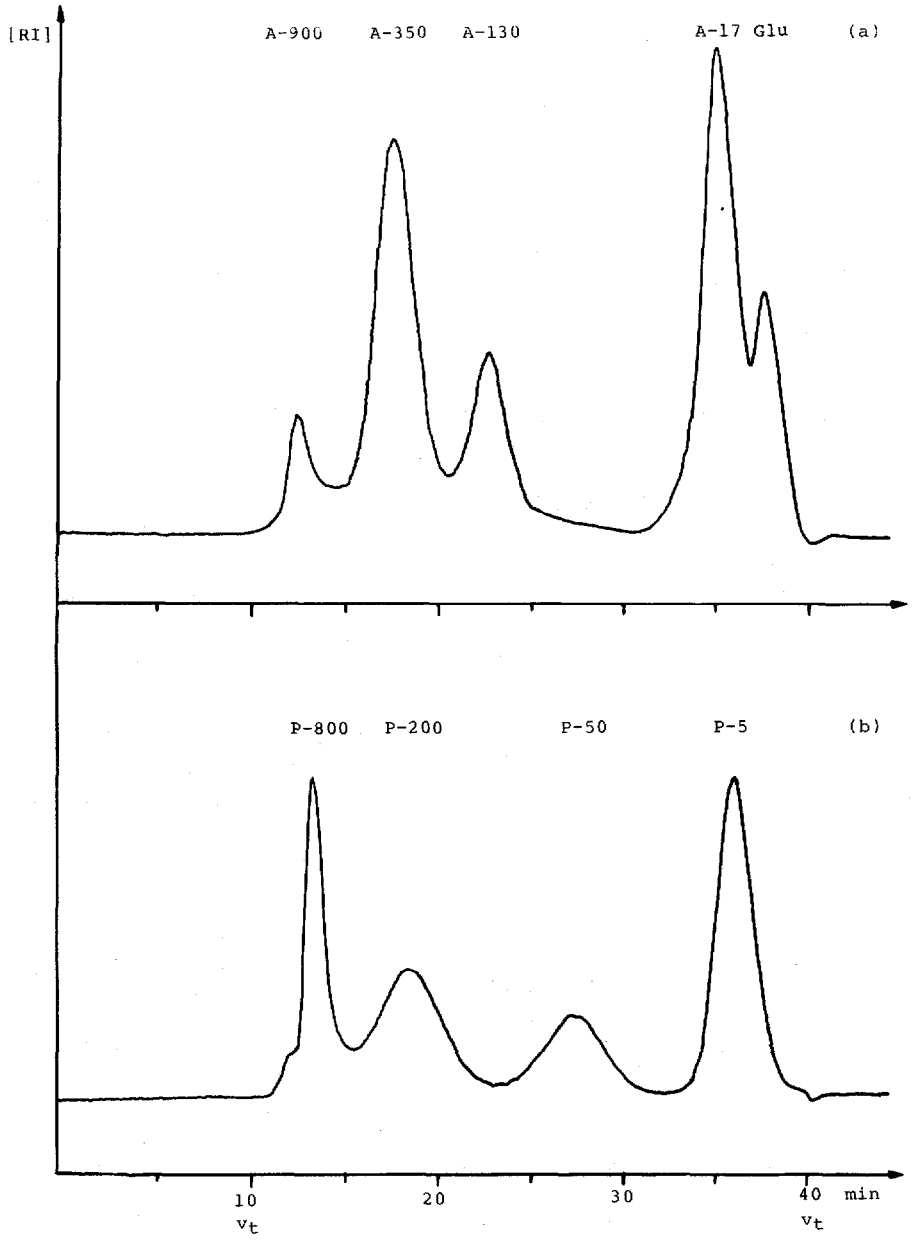

Fig. 1. HPGPC separation on Superose 6 HR10/30 of (a) amylose calibration standards and glucose and (b) pullulan calibration standards. For chromatographic conditions see Materials and methods.

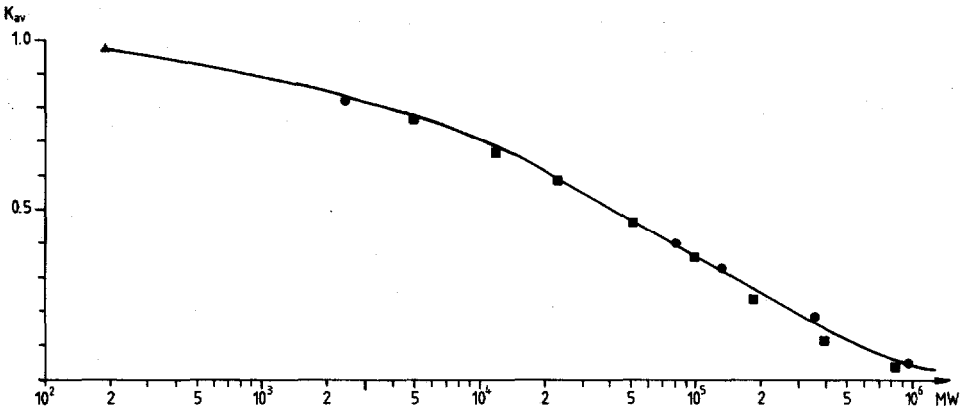

Fig. 2. Calibration graph for the Superose 6 column; relation between the distribution coefficient K_{av} and the molecular weight. Data points: ●, molecular defined synthetic amyloses; ■, pullulan standards P-82 (Shodex); ▲, glucose.

temperature and a flow-rate 0.6 ml/min. Calculation and calibration were carried out analogously to low-pressure GPC, as described by Praznik *et al.*[12]. The void volume was determined with dextran 5000 and amylopectin. The total volume of the system was determined with deuterium oxide.

RESULTS AND DISCUSSION

For some time agarose gels have been in common use for the separation and determination of starch polysaccharides in low-pressure GPC[1]. The Superose gel is also agarose-based, but cross-linked with epichlorhydrin, and a defined pore size and pressure stability distinguish this new type of gel. The narrow packing of the column also results in a high number of theoretical plates. The analytical Superose 6 column used also allows a quick separation with high resolution and excellent reproducibility. The fractionation range is for dextrans between $5 \cdot 10^3$ and $1 \cdot 10^6$ daltons, according to the producer's directions[20].

TABLE II

WEIGHT AND NUMBER AVERAGE MOLECULAR WEIGHT ($\overline{MW}_w$, $\overline{MW}_n$), WEIGHT AND NUMBER AVERAGE DEGREE OF POLYMERIZATION ($\bar{P}_w$, $\bar{P}_n$) AND DISPERSITY FACTOR ($\overline{MW}_w/\overline{MW}_n$) OF HYDROLYSED AMYLOSES

Duration of hydrolysis (h)	$\overline{MW}_w \cdot 10^{-4}$	$\overline{MW}_n \cdot 10^{-4}$	$\bar{P}_w$	$\bar{P}_n$	$\overline{MW}_w/\overline{MW}_n$
0	19.9	5.88	1230	360	3.42
4	7.13	3.50	440	215	2.05
8	5.11	2.61	315	160	1.97
16	3.33	1.96	205	120	1.71
32	2.10	1.31	130	80	1.62

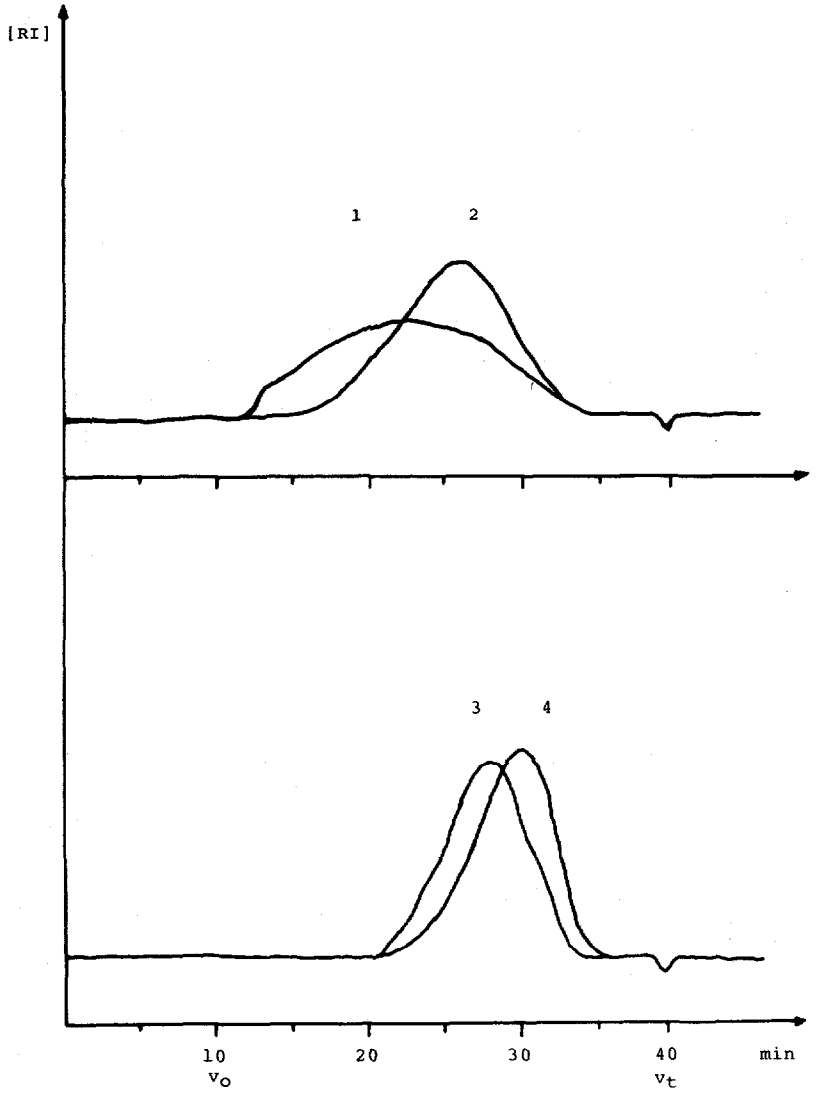

Fig. 3. HPGPC separation on Superose 6 HR10/30 of (1) Avebe potato amylose; (2) after 8 h hydrolysis; (3) after 16 h hydrolysis; (4) after 32 h hydrolysis. For chromatographic conditions see Materials and methods.

Because the GPC separation behaviour of polysaccharides is significantly influenced by their structure, it is necessary to calibrate the column with polysaccharides that have the same or at least similar structure[1,6]. Therefore we used molecularly defined amyloses and pullulans with a narrow distribution (Table I). Both polysaccharides are linear and structurally closely related, which is also shown by a similar migration behaviour in aqueous systems. Fig. 1 shows the chromatograms of these polysaccharide standards. The relation of the distribution coefficient K_{av} (calculated from the peak maxima) to the log of the molecular weight is shown in Fig. 2. No

significant differences in the migration behaviour of these two polysaccharide series were found. This makes it clear that either amylose or pullulan standards can be used for the calibration of GPC systems in which starch polysaccharides are to be determined.

Fig. 3 shows the chromatograms of hydrolysed amyloses. The amyloses produced by weak acidic hydrolysis tend to diminish the high- as well as the low-molecular-weight region during long reaction times. Table II lists the computed molecular parameters $\overline{MW}_w$, $\overline{MW}_n$, $\overline{P}_w$, $\overline{P}_n$, $\overline{MW}_w/\overline{MW}_n$. As can be seen from the dispersity factor $\overline{MW}_w/\overline{MW}_n$, there is an increase in the uniformity, which is why the low-molecular-weight portions, such as glucose, maltose or low-molecular-weight oligosaccharides, were removed during preparation of samples, and are therefore not taken into consideration for GPC analysis. In principle, a uniform statistical degradation of amylose is achieved that corresponds well with the acid-hydrolysis behaviour reported by Szejtli[21].

It is important to note that retrogradation, especially of low-molecular-weight amyloses, occurs readily in aqueous solution. To avoid this the samples should always be dissolved just before injection. Because the separation takes only 40 min and the sample is diluted on the column no retrogradation was noticed. The recovery rate was within the inaccuracy range of the detection system. The Superose 6 GPC system is therefore well suited for fast characterization of low-molecular-weight amyloses and starch degradation products.

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