ELSEVIER

Journal of Chromatography A, 768 (1997) 325–328

JOURNAL OF CHROMATOGRAPHY A

Short communication

# Calibration of a size-exclusion chromatography system using fractions with defined amylopectin unit chains

Helena Fredriksson*, Roger Andersson, Kristine Koch, Per Åman

*Department of Food Science, Swedish University of Agricultural Sciences, P.O. Box 7051, S-750 07 Uppsala, Sweden*

Received 5 November 1996; accepted 23 December 1996

**Abstract**

A high-performance size-exclusion chromatography (HPSEC) system was successfully calibrated using fractions of debranched amylopectin unit chains, obtained by gel filtration, and with the average chain length of the fractions determined by high-performance anion-exchange chromatography. As a comparison, calibration of the HPSEC system was also performed using maltoheptaose and pullulan standards as reference substances. The relationships between the degree of polymerization and the retention time for the defined amylopectin unit chain fractions and the commercial standards, i.e. maltoheptaose and pullulans, were very similar, indicating similar elution behaviour.

*Keywords:* Calibration; Molecular mass distribution; Amylopectin; Polysaccharides

## 1. Introduction

The length of the amylopectin unit chains may be of importance for the technological and nutritional properties of this polysaccharide. Size-exclusion chromatography is often used to analyze the molecular mass distribution of debranched amylopectin. The average degree of polymerization (DP) in collected fractions can be determined as the total carbohydrate concentration [1] divided by the reducing power [2]. Another method is to calibrate the columns by using various reference substances and to calculate the DP from a calibration curve. For an exact determination of the molecular mass distribution, it is necessary to use reference substances with narrow distributions and to cover the molecular mass range of the sample to be analyzed. It is also important to use references with a structure that is identical or very similar to that of the sample [3]. Pullulan standards, with an unbranched linear structure, consisting of (1→6)-linked α-maltotriose units, are often used as reference substances [4–7]. Maltooligosaccharides and amylose [8] as well as dextrans [9,10] have also been used for calibration. A convenient method was introduced where a laser-light-scattering photometer and a differential refractometer (RI), connected in sequence, monitors the molecular mass directly [11]. High resolution of individual peaks for linear (1→4)-linked α-D-glucan, with a DP between 6 and 60, can be obtained by using high-performance anion-exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) [12–15].

In this study, a high-performance size-exclusion chromatography (HPSEC) system was calibrated by

*Corresponding author.


*PII* S0021-9673(97)00039-3

using fractions with defined amylopectin unit chains. The average chain length in the fractions was determined by HPAEC. This calibration was compared with that obtained with the commonly used pullulan standards and maltoheptaose.

## 2. Experimental

### 2.1. Chemicals and enzymes

All chemicals used were of analytical grade. Sodium acetate was purchased from Fluka (Buchs, Switzerland) and sodium hydroxide, 50% solution, was from J.T. Baker (Deventer, Netherlands). Maltoheptaose (Boehringer Mannheim, Mannheim, Germany) and pullulans; P-5, P-10 and P-20 with DPs of, 35.8, 75.3 and 146.3, respectively (Macherey-Nagel, Düren, Germany), were used as reference substances. Isoamylase (EC 3.2.1.68) from *Pseudomonas amyloderamosa*, with an activity of 71 000 U/mg protein was obtained from Hyashibara Biochemical Labs. (Okayama, Japan). Water from a Milli-Q water purification system was used. All eluents were filtered (0.45 μm) and degassed before being used in chromatography.

### 2.2. Preparation of amylopectin unit chain fractions

Waxy maize starch was debranched with isoamylase, essentially according to Lloyd et al. [7]. A 20-mg sample was incubated in 1.45 ml of 0.06 *M* acetate buffer (pH 3.6) and 5 μl of isoamylase for 2.5 h at 38°C. Thereafter the enzyme was inactivated by heating in a boiling water bath for 5 min. Debranched amylopectin was fractionated by gel permeation chromatography on a Bio-Gel P-6 column (75×1.6 cm) (Bio-Rad, Richmond, CA, USA) using water as the eluent at a flow-rate of 0.4 ml/min. The elution profile was monitored by refractive index (RI detector R-403, Waters Associates, Milford, MA, USA) and 2-ml fractions were collected.

### 2.3. Determination of average DP in amylopectin unit chain fractions

The average chain length of the amylopectin unit chain fractions was determined by a HPAEC system with a gradient pump (GP40, Dionex, Sunnyvale, CA, USA), and a PAD system (ED40, Dionex). The system was connected to an autosampler with a 20-μl loop (SpectraSYSTEM AS 3000, Spectra-Physics, Fremont, CA, USA). The pulsed potentials and durations were $E1 = +0.05$ V ($t = 480$ ms), $E2 = +0.60$ V ($t = 120$ ms) and $E3 = -0.80$ V ($t = 300$ ms). A Dionex CarboPac PA-100 column (250×4 mm) and a PA-100 guard column were used. Eluents A and B were 150 m*M* sodium hydroxide and 150 m*M* sodium hydroxide containing 500 m*M* sodium acetate, respectively, at a flow-rate of 1 ml/min. The gradient program was as follows: 66% of eluent A at 0 min, 55% at 5 min, 33% at 55 min, 10% at 80 min, 66% at 81 min and thereafter isocratic for 15 min. A 20-μl sample was injected and the analysis was performed at room temperature. All samples were filtered through a PTFE filter (0.45 μm) before analysis.

The average DP of each fraction was calculated as:

$$\text{Average DP} = \frac{\sum_{i=1}^{n}(A_i N_i)}{\sum_{i=1}^{n} A_i}$$

where $n$ = number of peaks, $A_i$ and $N_i$ = peak area and DP, respectively, for peak $i$.

Maltoheptaose was used to determine the retention time of the peak with a DP of seven. For the following peaks, DP was estimated assuming a homologous series, adding one glucose residue for each peak. The first fraction from the gel filtration with enough resolution to calculate the average DP had an average DP of 59.

### 2.4. Calibration of HPSEC

The HPSEC system consisted of a pump (No. 2248, LKB, Bromma, Sweden) set at a flow-rate of 0.45 ml/min, an injector (Model U6K, Waters Associates) and a RI-detector (Model 132, Gilson, Villiers le Bel, France). A series of columns with TSK-gels; one with G3000 SWXL and two with G2000 SWXL (each 300×7.8 mm) and a SWXL guard column (TosoHaas, Stuttgart, Germany) were used. The amylopectin unit chain fractions, with

average DPs ranging from 6 to 59 (as determined by HPAEC), were injected (50–100 μl) onto the column after filtering through a PTFE filter (0.45 μm) and were eluted at room temperature with 0.01 *M* sodium acetate buffer (pH 5.0). The HPSEC system was also calibrated using maltoheptaose and pullulans (0.5 mg/ml) with defined molecular masses as the reference substances.

## 3. Results and discussion

Waxy maize starch was debranched with isoamylase and fractionated by gel filtration. Collected fractions were analyzed with HPAEC and the average chain length of each fraction was calculated from individual peaks with known DP (Fig. 1). The accuracy of the calculated average DP was reduced for fractions with high molecule mass due to low resolution (DP 52 and 59). The amylopectin unit chain fractions were then analyzed by HPSEC to get the retention time of their peak maximum (Fig. 2). A linear relationship often exists between retention times ($t_R$) and the logarithms of the molecular masses [16]. The separation mechanism is based on solution size hydrodynamic volume, and not molecular mass, and, therefore, the system must be calibrated with standards of the same or similar chemical structure [3]. When the often used maltoheptaose and pullulan standards were analysed, their retention times followed the same equation ($DP = 3796.35 + 50.23t_R - 872.52t_R^{0.5}$) as those obtained for the amylopectin unit chain fractions (average DP 6–59). Regression including all data points gave a standard error of 1.9 (Fig. 3). This result shows that the commonly used pullulan standards [4–7] exhibit an elution behaviour that is similar to that of the amylopectin chains, despite the difference in chemical structure.

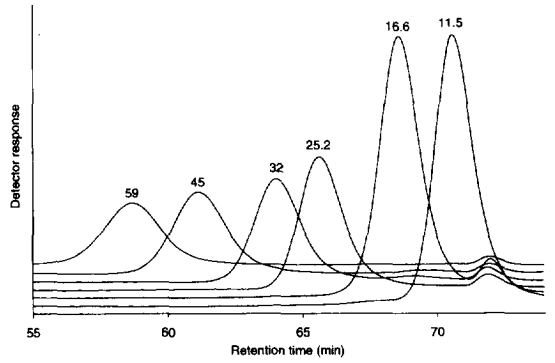


Fig. 2. Selected chromatograms obtained by HPSEC for amylopectin unit chain fractions. Average DP is indicated above each peak.

The main advantage of the described method for the calibration of a HPSEC system is that the large number of calibration fractions provided an accurate description of the non-linear relationship between DP and retention time. The calibration was also performed with unit chain fractions of the same origin and structure as that of the samples, in this case amylopectin unit chains, to be analyzed. One limitation is that only fractions with individual amylopectin unit chains with an average DP of up to about 60

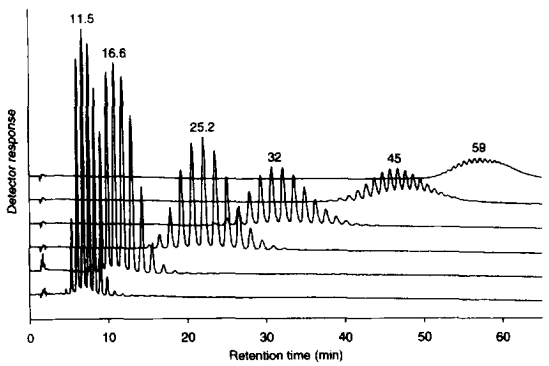


Fig. 1. Selected chromatograms obtained by HPAEC for amylopectin unit chain fractions. Average DP is indicated above each fraction.

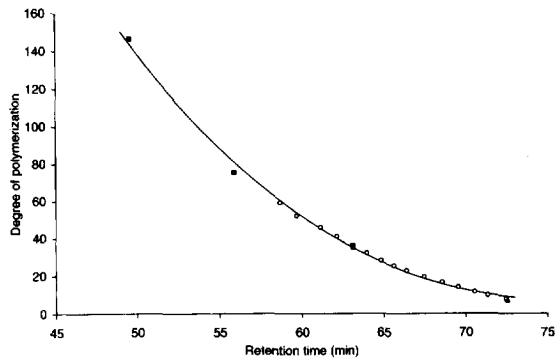


Fig. 3. Relation between peak retention time obtained by HPSEC and the average DP of amylopectin fractions (○), maltoheptaose (▲) and pullulan standards (■).

are available as calibration substances, due to the poor resolution of the high-molecular-mass material by the HPAEC.

The results of this study show that the described method proved to be useful for calibration of HPSEC and can be used as an alternative method to detection with light scattering, which requires advanced equipment that is not generally available. Upon comparison, the defined amylopectin unit chain fractions, maltoheptaose and the pullulan standards showed a similar relationship between retention time and DP.

## Acknowledgments

Financial support was obtained from The Swedish Council for Forestry and Agricultural Research and collaborating Swedish food industry.

## References

[1] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers and F. Smith, Anal. Chem., 28 (1956) 350.
[2] N. Nelson, J. Biol. Chem., 153 (1944) 375.
[3] L.L. Lloyd, J.F. Kennedy and C.J. Knill, in W. Brown (Editor), Light Scattering Principles and Development, Vol. 1, Clarendon Press, Oxford, 1996, pp. 494–523.
[4] W. Zhang and D.S. Jackson, J. Food Sci., 57 (1992) 1428.
[5] L.Z. Wang and P.J. White, Cereal Chem., 71 (1994) 263.
[6] P. Cairns, L. Sun, V.J. Morris and S.G. Ring, J. Cereal Sci., 21 (1995) 37.
[7] J.R. Lloyd, C.L. Hedley, V.J. Bull and S.G. Ring, Carbohydr. Polym., 29 (1996) 45.
[8] S. Kobayashi, S.J. Schwartz and D.R. Lineback, Cereal Chem., 63 (1986) 71.
[9] A.-C. Salomonsson and B. Sundberg, Starch, 46 (1994) 325.
[10] P. Lehtonen, Chromatographia, 26 (1988) 157.
[11] S. Hizukuri, Carbohydr. Res., 141 (1985) 295.
[12] R.N. Ammeraal, G.A. Delgado, F.L. Tenbarge and R.B. Friedman, Carbohydr. Res., 215 (1991) 179.
[13] K. Koizumi, M. Fukuda and S. Hizukuri, J. Chromatogr., 585 (1991) 223.
[14] Y.-C. Shi and P.A. Seib, Carbohydr. Res., 227 (1992) 131.
[15] A. Susuki, M. Kaneyama, K. Shibanuma, Y. Takeda, J. Abe and S. Hizukuri, Cereal Chem., 69 (1992) 309.
[16] H. Determann, in J.C. Giddings and R.A. Keller (Editors), Advances in Chromatography, Vol. 8, Marcel Dekker, New York, 1969, pp. 3–45.