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Molecular characterization of starch by SEC: dependance of the performances on the amylopectin content

Yfeng Chen, Christophe Fringant and Marguerite Rinaudo

Centre de Recherches sur les Macromolécules Végétales (CERMAV-CNRS) BP 53, 38041 Grenoble Cedex 9, France

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The solubility of starch samples with different amylose/amylopectin ratios was investigated in NaOH and aqueous DMSO solutions. The decrease in solubility when the amylopectin content increases was clearly demonstrated. SEC experiments were performed using KOH 0.001 N as it was found to be better than the other aqueous solvents investigated. Even when the sample solubility was found to be high, SEC experiments showed the occurrence of some aggregation as a result of the retrogradation process. This phenomenon is undesirable for quantitative use of SEC and could not be diminished by the use of various additives. This paper demonstrates that no aqueous solvent tested is suitable for characterizing starch amylopectin and that the use of this technique has to be limited to the characterization of the amylose component of starch. © 1997 Elsevier Science Ltd

INTRODUCTION

Starch is a natural neutral polysaccharide. Its repeating unit is the D-glucose residue linked mainly by α-(1-4) linkages. In fact, it has been known for a long time that starch is composed of two macromolecules differing both in size and structure (Schoch, 1942). Amylose is a weakly branched polymer (Bank & Greenwood, 1966) which has typically a molecular weight of $\sim 10^5$. Amylopectin is a highly branched polymer (Manners, 1989) and its molecular weight is in the range $5*10^6$–$5*10^7$. Many studies have been made to determine the structures of amylose and amylopectin from various botanical origins. In these studies, gel chromatography has been used in three different ways.

After separation of amylose from amylopectin by selective complexation, the purity of the amylose fraction is checked (Takeda *et al.*, 1984) by collecting fractions after filtration on various gels and determining the carbohydrate content in the eluted solution.

Another use of gel chromatography is to determine the size and the different populations of chains obtained after debranching of amylopectin by isoamylase (Ong *et al.*, 1994).

Surprisingly, the use of gel permeation chromatography for direct determination of molecular weight distribution has not been widely developed. Considering the molecular differences between amylose and amylopectin, it should be possible to obtain a direct fractionation of both components and the use of combined detectors should provide direct molecular weight distribution. This has been done for certain amylose samples (Van Djik *et al.*, 1976; Hizukuri, 1984).

In the case of native starch, the main problem encountered was the search for a good solvent. Different solvents were proposed in the literature such as DMSO (Hizukuri, 1985; Manners & Matheson, 1981; Salemis & Rinaudo, 1984a) for steric exclusion chromatography (SEC) or aqueous alkaline solution (1 N NaOH or further diluted) (Hizukuri *et al.*, 1981; Szejtli *et al.*, 1967), acidic conditions (Yamada & Taki, 1976; Biliaderis *et al.*, 1979) or DMAc/LiCl (Politz *et al.*, 1994). However, the results obtained are not very conclusive and the authors often report some problems in performing a quantitative analysis. In our opinion, the most interesting results up to now for native starch were obtained by Yamada & Taki (1976). They performed GPC of starches on Sepharose 2B columns in acidic aqueous medium. A good separation was observed between amylose and amylopectin.

In this work, we focused on the use of aqueous solvents and more especially we demonstrated that the main parameter is the lack of solubility of the amylopectin component. The solubility of different starch samples is investigated in relation to their composition and SEC experiments are performed to establish the molecular weight distribution.

MATERIALS AND METHODS

Different samples of starch were analysed. Collys is a commercial corn starch from Roquette (France) characterized by a high amylose content. Waxilys (Waxy) is a nearly pure amylopectin maize starch from Roquette. Avebe is a high amylose starch extracted from potato by a liquid–liquid process and purchased from Avebe (Holland). Pea amylose was prepared in our laboratory by aqueous leaching and selective complexation of amylose with *n*-butanol. A linear amylose obtained by enzymic synthesis was kindly provided by S. Kitamura (Kyoto, Japan); it is used as reference and was found to be remarkably soluble in water.

Before analysis, the starch granules were disrupted by dissolution in pure DMSO followed by a filtration and reprecipitation by ethanol. This procedure enabled starch samples to be obtained free from any insoluble residue. Such samples will be called 'destructured' in the rest of the paper.

In order to increase the amylose content of some of our starch samples, recrystallization of amylose was performed by selective precipitation with *n*-butanol. A solution of destructured starch (1 g) in DMSO (5 ml) was diluted to 500 ml with water, then heated to 80°C under nitrogen, and *n*-butanol (20 g) was added. The solution was stored at 4°C for 24 h allowing the complex to crystallize. It was then isolated by centrifugation and dissolved in boiling water under nitrogen. The complex was reprecipitated by the above procedure. The obtained complex was washed three times with ethanol, and then with acetone and ether successively. The absence of any residual trace of butanol was checked by NMR. We did not perform many recrystallizations, as our objective was not to obtain pure amylose. These samples are called 'crystallized' in the rest of the paper.

The solubility of starch in the different solvents was determined by filtration through a porous membrane (pore diameter 0.2 μm). The exact concentration of polymer in solution (before and after filtration) was determined by the method proposed by Dubois (phenol and sulphuric acid). The percentage of solubility was calculated according to:

$$\%\,\text{solubility} = (C_f/C_i) \times 100$$

where C_f and C_i are the concentrations of polymer in solution after and before filtration, respectively.

The amylose content was estimated by the weight of iodine bound by 100 g of materials (CLI). For linear amylose the CLI equals 20 (Bank *et al.*, 1971, Takeda *et al.*, 1992). For highly branched amylopectin, the CLI is close to zero. The starch sample was dissolved in DMSO (10 mg/ml). The solution was then diluted with water to obtain a final concentration of 1% (v/v) in DMSO and potassium iodide (0.05 N) was added. The formation of the complex was obtained by successive addition of an aqueous solution of iodine (1 mg/ml I_2, 0.05 N KI, 1% DMSO). The bound iodine was deduced from determination of the quantity of free iodine in solution by spectrophotometric measurements of the absorbance at 288 nm.

The determination of intrinsic viscosities of the different sample was made in 1 N KOH using a capillary viscometer (Viscomatic MS, Fica) thermostated at 25°C. The molecular weight were estimated using the Mark–Howink relationships:

$[\eta]_{\text{amylose}} = 1.18\times10^{-3}\,M_w^{0.87}$ (Cowie, 1961).

$[\eta]_{\text{amylopectin}} = 2.16\times10^{-3}\,M_w^{0.67}$ (Salemis & Rinaudo, 1984b).

SEC experiment were performed on grafted silica columns (Shodex: Ionpak S-801, S-804, S-805). This combination of columns allows the separations of molecules with a wide range of molecular weights (from simple sugars to high molecular weight polymers). An example of separation obtained with a mixture of oligosaccharides is presented in Fig. 1. The output of the columns was connected in line to a light scattering detector (Chromatix KMX-6) and to a differential refractometer (Waters R401). All the solutions were filtered on a porous membrane (0.2 μm pore diameter) before injection. The eluent was 0.001 N KOH.

RESULTS AND DISCUSSION

In order to perform high quality SEC experiments, it is very important to prepare a good solution of the

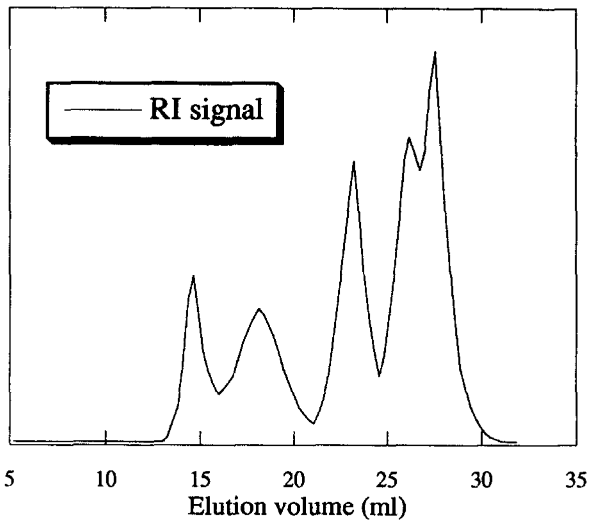

Fig. 1. Elution profile of a mixture of carbohydrates: synthetic amylose (V_e ~14.5 ml), dextran (V_e ~18 ml), malodextrin (V_e ~26 ml), lactose (V_e ~26 ml) and glucose (V_e 28 ml).

Table 1. Solubility of different starch samples in water/DMSO (99:1) mixture (concentration 0.05 mg/ml)

	Avebe crystallized	Waxilys destructured	Collys destructured	Collys crystallized
Solubility (%)	98	47	78	89
CLI (g/100 g)	17.5	0.04	14.6	16.0

sample. For this purpose, we first investigated the solubility of various starch samples in aqueous solvents.

Solubility in aqueous DMSO (1% concentration)

The destructured starch samples were first dissolved in pure DMSO to give a perfect solution (with stirring and moderate heating if necessary). Then, this solution (5 mg per ml DMSO) was diluted with water to 1% DMSO. The final concentration of polymer was around 5×10^{-2} mg/ml. The concentration of this solution was determined precisely using the Dubois method and then filtered. The soluble fraction was determined in the filtrate allowing the estimation of solubility.

The solublities of various samples are given in Table 1. The amylose content for each sample as reflected by the CLI index is also given in Table 1. From these data, it seems that solubility decreases when amylopectin content increases.

In order to confirm the influence of amylopectin content on solubility, we prepared some synthetic mixtures with different ratios of recrystallized Avebe (amylose rich) and destructured Waxilys (amylopectin). We determined the solubilities of these mixtures in 1% DMSO and the CLI index of the solution before and after filtration. The results are reported in Table 2. From these data, it is obvious that the solubility decreases while amylopectin content increases. Moreover, the value of the CLI index after filtration is higher than before filtration. This clearly indicates that the insoluble part retained on the membrane is amylopectin rich.

The role of the DMSO concentration was also considered as well as the starch concentration; the results are given in Table 3. From these data, we confirm that the solubility depends not only on the DMSO content of the solvent, but also on the polysaccharide concentration. Maximum solubility is obtained at the lowest polysaccharide concentration. It is clear that water/DMSO mixtures are not good solvents for starch: the amylopectin fraction has a low solubility even in very dilute solutions.

Solubility in NaOH

The solubility of the different starch samples was studied for different polymer concentrations in NaOH 1N. The results are given in Table 4. It can be seen from this table that the solubility is better than in 1% DMSO even for amylopectin rich starch when the polymer concentration is sufficiently low. After filtration, the dilution of these solutions with water gives clear solutions without any precipitation of a fraction of this initially soluble sample. This allows us to propose the following procedure for further characterization by steric exclusion chromatography: sample solubilization in 0.1 N KOH (or NaOH), dilution with water to 0.01 N KOH and use of 0.001 N KOH as eluent.

Nevertheless, the previous data show that it is impossible to obtain a complete solubilization of amylopectin whatever the polymer contration is. The conclusion is that it is impossible to characterize quantitatively any starch sample by aqueous SEC.

Table 2. Solubility and CLI of synthetic mixtures with varying amylose (Avebe)/amylopectin (Waxy) ratios

Avebe ratio (%)	Solubility (%)	CLI before filtration (g/100 g)	CLI after filtration (g/100 g)
100	96	17.5	17.9
80	85	13.7	15.3
50	66	9.9	13.0
20	42	3.6	4.1
0	26	0.04	—

Table 3. Dependence of the solubility of destructured Collys on the solvent composition and the polymer concentration in aqueous DMSO

Concentration of DMSO in the solvent (%)	Concentration of polymer (mg/ml)	Solubility (%)
100	5	98
90	2	58
90	0.5	91
50	2.5	73
50	0.5	81
1	0.05	78

Table 4. Solubility of different starch samples in 1 N NaOH

Starch sample	Solubility ($C = 1$ mg/ml)	Solubility ($C = 0.1$ mg/ml)
Avebe crystallized	99	99
Waxilys destructured	20	64
Collys destructured	79	93

Molecular weight analysis

The starch samples were dissolved in 0.1 N KOH and diluted to obtain a polymer concentration in the range 1 mg/ml in 0.01 N KOH. The sample was filtered before injection on 0.2 μm porous membrane. The true polymer concentration was also determined by the Dubois method before and after filtration to determine the solubility. The quantities of polymer injected are reported in Table 5. For each sample, it can be seen that there is no significant difference between the real quantity of polymer injected, and the one calculated using the refractometer signal. This is the case whatever the starch sample is and it allows us to conclude that there is no adsorption on the colunms. All the material injected is eluted through the columns but it is only partially representative of the starch as only a fraction of the sample (depending on the amylopectin content) can be solubilized.

As retrogradation of amylose in aqueous solution is well known, we investigated the stability of the solution in different solvents. For this purpose, the solutions were injected immediately after being prepared or allowed to stand at room temperature for 24 h. The solutions were then filtered and injected. In all cases, the eluent 0.001 N KOH was retained. The chromatograms are presented in Fig. 2. It can be seen that the area of the refractometer signal (that is directly related to the quantity of polymer eluted) is always smaller for the solution after 24 h than when injected immediately after

Table 5. Role of the starch origin in its solubility and relation with elution yield

Starch sample	Mass of polymer calculated from refractometer SEC detector (μg)	Mass of polymer after filtration determined by Dubois (μg)	Solubility (%)
Avebe destructured	76.96	79.67	89.1
Collys destructured	63.08	63.22	70.0
Waxy destructured	29.98	35.01	39.5
Pea amylose	72.78	73.87	83.0
Synthetic amylose	85.11	87.13	94.3

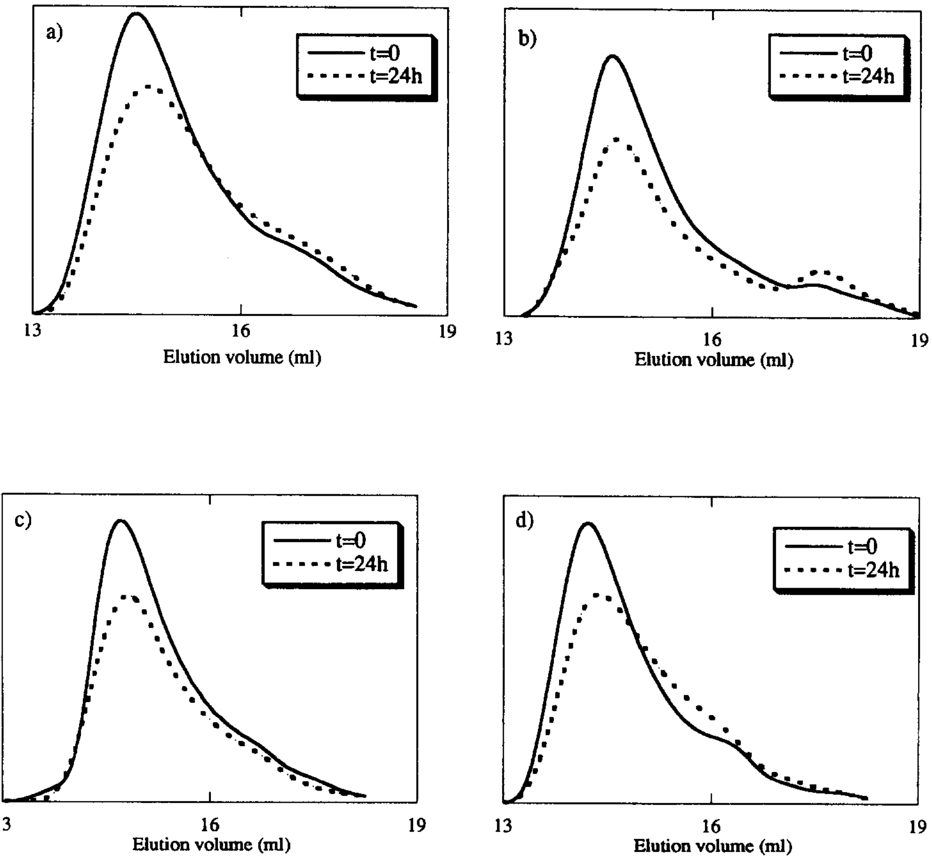

Fig. 2. Elution profile of a solution of synthetic amylose immediately after dissolution ($t = 0$) and after 1 day ($t = 24$ h) with different solvents: (a) 0.1 N NH_4NO_3, (b) 0.001 N KOH, (c) 0.001 N KOH and 2% glycerol, (d) 0.25 N CH_3CO_2Na.

being prepared. This indicates that, after a long time, a process of aggregation and/or retrogradation occurs, resulting in the formation of a structure retained during filtration. This means that even when studying the molecular characteristics of amylose, although there is no problem of solubility, it is necessary to take care and to work with freshly solubilized samples to make a quantitative analysis.

The chromatograms obtained for different starch are presented in Fig. 3. The light scattering signal allows the molecular weight distribution of the eluted fraction to be determined. This, of course, clearly does not represent the whole sample. The weight average molecular weight (M_w) of the samples have been calculated using the true quantity of starch eluated. The results are given in Table 6. The comparison with the values of the viscosity average molecular weight estimated from intrinsic viscosity measurements gives good agreement (usually $M_v < M_w$ for $a < 1$) except in the case of destructured Collys. This is explained by the coefficients of the Mark–Houwink relationship used for the calculation which are different for amylose and amylopectin. In the case of Collys, both polymers are present in the solution but we used the coefficients proposed for amylose.

In the case of amylopectin, both values of molecular weights are in good agreement, but the low solubility of this polymer makes it impossible to consider the obtained values as representative of the sample.

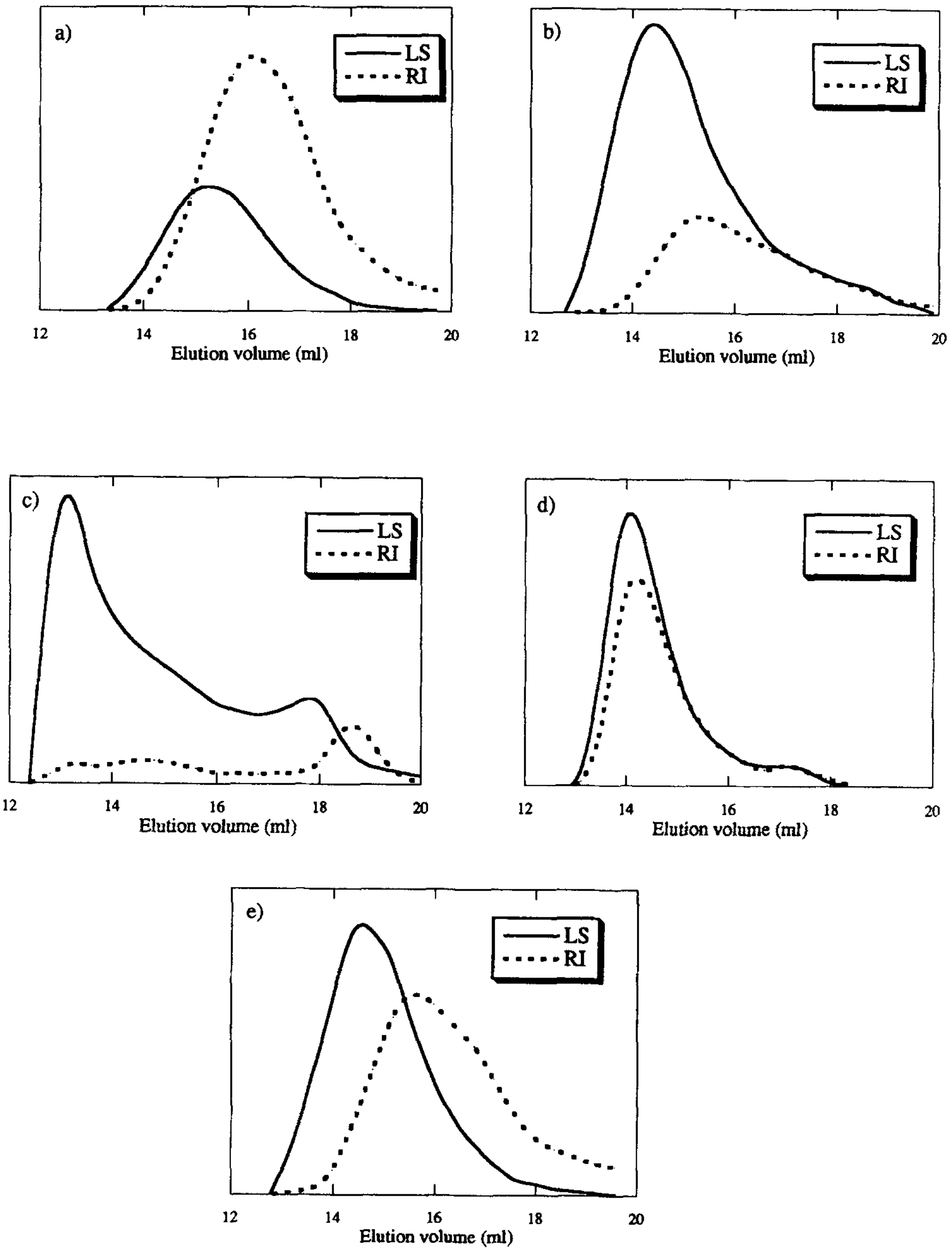

Fig. 3. Chomatograms of various starch samples (RI is the refractometer signal and LS is the light scattering signal): (a) destructured Avebe, (b) destructured Collys, (c) destructured Waxilys, (d) synthetic amylose, (e) pea amylose.

Table 6. Characteristics of different starch samples determined by SEC and viscosimetry experiments

Starch sample	M_w (10^5 g/mol)	M_n (10^5 g/mol)	i (M_w/M_n)	M_v (10^5 g/mol)	[η] (ml/g)
Synthetic amylose	8.15	7.78	1.05	8.48	
Pea amylose	6.48	2.64	2.45	7.7	206
Avebe destructured	2.6	1.64	1.58	3.75	108
Collys destructured	13.6	8.14	1.67	3.6	106
Waxy destructured	191	45	4.24	175	154

In these aqueous solvents, only the starch rich in amylose can be correctly analysed; some of the amylopectin is retained on the filter as large particles.

CONCLUSION

In this paper, we investigated the solubility of different starches in aqueous solvents (1% DMSO or NaOH, 10^{-2} or 1 M KOH). The solubility is shown to decrease in direct relation to the amylopectin content.

Steric exclusion chromatography which require a good solvent only fractionates the soluble fraction of starch, namely the linear amylose. Finally, a kinetic process of aggregation is demonstrated. It is suggested that the best solubilization procedure prior to SEC is to prepare a dilute solution (1 mg/ml or less in 0.1 M NaOH or KOH) which is diluted to 10^{-2} M NaOH or KOH and directly injected.

This work demonstrates again that it is very difficult to determine the molecular weight distribution of starch.

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