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Characterization of storage cell wall polysaccharides from Brazilian legume seeds and the formation of aqueous two-phase systems

T.T. Franco[a,*], N.R. Rodrigues[a], G.E. Serra[a], V.R. Panegassi[a], M.S. Buckeridge[b]

[a]CPQBA-UNICAMP, caixa postal 6171, Campinas 13081-970, Brazil
[b]Instituto de Botânica, caixa postal 4005, São Paulo 01051-000, Brazil

Abstract

Cell wall storage polysaccharides from Brazilian legume seeds of *Dimorphandra mollis, Schizolobium parahybum* (galactomannans), *Copaifera langsdorffii, Hymenaea courbaril* (xyloglucans) and the galactan from cotyledons of the Mediterranean species *Lupinus angustifolius* were extracted and their apparent molecular masses were determined by high-performance size exclusion chromatography analysis. They were, to a large degree, polydisperse, showing molecular masses that varied from 100 000 to 2 000 000. Polyethylene glycol (PEG, 1500, 4000, 6000 and 8000), sodium citrate and dextran (73 000, 60 000–90 000, 505 000 and 2 000 000) were used for investigating phase formation with the seed polysaccharides. Galactomannans and xyloglucans demonstrated phase formation with sodium citrate concentrations lower than 30%, as well as dextrans and polyethylene glycol, and formed gels in the presence of high concentrations of sodium citrate (above 30%). Galactan did not promote phase formation with any of the reagents used. On the basis of the results obtained, the possibility of using legume seed polysaccharides for the partitioning and purification of polysaccharide enzymes in aqueous two-phase systems is suggested.

Keywords: Aqueous two-phase systems; Legume seeds; Polysaccharides; Saccharides

1. Introduction

Leguminous seeds store cell-wall polysaccharides (CWSP) of several types in their cotyledons or in the endosperm. In such cases, CWSP can constitute 40% or more of the dry weight of the seed, and specialized (thick) cell walls are present containing massive amounts of a single cell wall component. There are three main groups of major CWSP, namely the mannans group (which is composed of galactomannans, "pure" mannans and glucomannans), xyloglucans and galactans [1,2].

Galactomannans are polysaccharides derived from the endosperm of plant seeds. Their structure is usually based on a 1,4-linked backbone of β-D-mannopyranosyl residues having side chains linked α-D-(1,6) and consisting of single α-D-galactopyranosyl groups (Fig. 1a). In Leguminosae, the mannose/galactose ratio and the statistical distribution of galactosyl residues along the mannan backbone vary from species to species [3–5]. The degree of branching by galactose in galactomannans is thought to affect water solubility. As branching decreases, the more insoluble the galactomannan becomes and molecules containing less than 10% galactose will precipitate [6].

Seed xyloglucans have a cellulose-like β-D-(1,4)-glucan backbone to which single-unit α-D-

*Corresponding author.

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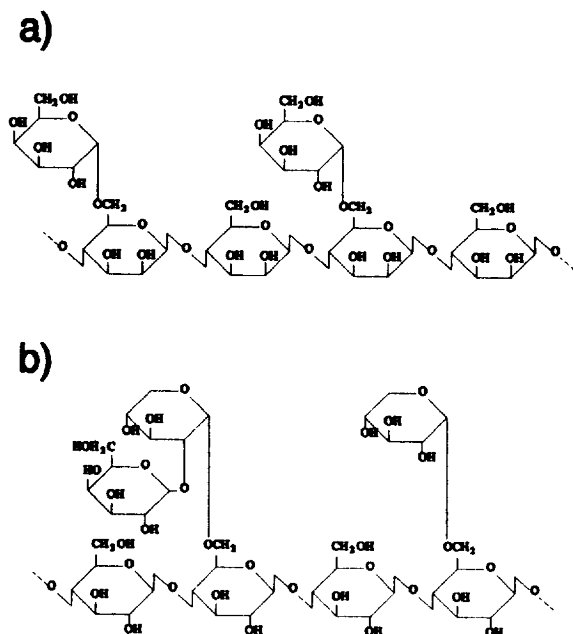

Fig. 1. Chemical structure of polysaccharides. (a) Galactomannan; (b) xyloglucan.

xylopyranosyl substitutes are attached (Fig. 1b). Some xylosyl residues are further substituted at O-2 by β-D-galactopyranosyl residues [7]. The pattern of xylose substitution is remarkably regular, with virtually the entire molecule being composed of repetitive units of glucose 4:xylose 3 with variable galactose substitution [8].

Hirst et al. were the first to identify the galactan in seeds of the white lupin (*Lupinus albus*) as a distinct polysaccharide [9]. Recent work, based on chemical structural determinations including methylation and enzymatic analysis, suggests that the *Lupinus angustifolius* polysaccharide is a β-(1,4)-linked D-galactan which consists of branches of a core molecule of rhamnogalacturonan [6].

Aqueous two-phase systems (ATPS) have been used as an alternative procedure to separate and purify proteins on a large-scale and are recommended for the entire range of biochemical separations of microbial enzymes and macromolecules either in batch or in continuous processes [10–12]. They can replace the initial steps in protein purification and chromatography and be scaled up, without appreciably changing the nature, or efficiency, of the process [13]. Conventionally, polysaccharide-degrading enzymes are purified by a sequence of steps in a tedious and time-consuming operation, using ion-exchange and affinity chromatography [6]. Using a PEG/dextran system, Aurol [14] has purified and recovered the enzyme, dextran-saccharase, in the bottom, dextran-rich phase, whereas the main contaminants (proteases and pigments) were collected in the top, PEG-rich phase, in a single-step operation. By using the same principle of affinity interaction between the polysaccharide degrading enzyme and the substrate, the enzyme α-amylase from *Clostridium thermsulfurigenes* was purified on a raw starch column.

In general, and particularly on a laboratory scale, PEG–dextran and PEG–phosphate systems were most frequently used. The phases are compatible with most of the known proteins and provide a gentle environment for biological materials, since both phases are composed primarily of water. Phase separation of two structurally different polymers occurs as a result of the hydrogen bonding of polymers with water, resulting in repulsive interactions when one phase is enriched with one polymer while the other is enriched with respect to the second polymer. In polymer–salt systems, one phase is enriched with a polymer and the other with salt [15]. As partitioning in ATPS is mainly a process in which the exposed groups come in contact with the phase components, it is possible to have a selective partitioning of biomolecules using polymer systems, based on the molecular surface characteristics of the compounds to be partitioned. The influence of different factors upon the partitioning of proteins is considered mainly to be the result of electrostatic, hydrophobic and biospecific interactions [16–20].

The most common components of ATPS are the synthetic PEGs (of different molecular masses) and the microbial dextrans [(predominantly poly (α-1,6-D-glucose)]. For large-scale protein purification, dextran is considered expensive and therefore, it would be desirable to find alternatives. Crude dextran was successfully employed as a substitute for fractionated dextrans on the purification and recovery of the enzymes pullulanase and formate dehydrogenase [21]. Tjerneld et al. [22] developed a method to replace the latter by chemically modified starches that were demonstrated to have desirable properties for the partitioning of β-galactosidase and phospho-

fructokinase. Venancio and Teixeira [23] have evaluated the crude hydroxypropyl starch (HPS), already used in the paper, food and textile industries, for the formation of polymer–polymer systems with PEG. HPS was succesfully employed for the partitioning of ovalbumin and bovine albumin, as well as for cells.

As there is still a need to develop new ATPS suitable for separation processes, as the purification of polysaccharide degrading enzymes is usually tedious and, as legume seed polysaccharides seem to be an attractive source of cheaper and more abundant material, we have selected five newly discovered polysaccharides to be further characterized. The aim of the present work was to investigate the molecular masses and phase formation of two galactomannans and two xyloglucans from Brazilian legume seeds and a galactan from a Mediterranean species.

2. Experimental

2.1. Plant polysaccharide extraction

Galactan (G 1) was extracted from the catyledons of *Lupinus angustifolins* as described by Buckeridge and Reid [6]. Galactomannans were extracted from the seeds of *Schizolobium paraibum* by breaking the seeds and separating the endosperm manually. The ground endosperm was designated fraction G2.

Galactomannan was extracted from *Dimorphandra mollis* seeds, through a combination of milling, grinding and sieving the de-coated seeds. De-coating was performed by 5% NaOH extraction. After sieving, the 420 mesh powder was collected. This was called fraction G3 and was subjected to water extraction for 6 h at 80°C, followed by precipitation with 5 vol. of ethanol. The precipitate was collected, solubilized in hot water and freeze-dried to give fraction G4.

Xyloglucans were extracted from the powdered seeds from *Copaifera langsdorffii* and *Hymenaea courbaril* with 2% NaOH for 2 h at 60°C. After neutralization with acetic acid, the filtrate was precipitated with 3 vol. of ethanol. The precipitate was collected, solubilized in hot water and freeze-dried. These were called fractions X1 and X2, respectively.

For phase separation and high-performance size exclusion chromatography (HPSEC) analysis, the polysaccharides were solubilized in water containing sodium azide (0.02%) and then were heated in a water bath at 45°C, under continuous stirring.

The dextran commercial samples were from Sigma (St. Louis, MO, USA; M_rs: 73 000, 60 000, 90 000; 505 000 and 2 000 000). PEG of M_rs of 1500, 4000, 6000 and 8000 were from Synth (Campinas, SP, Brazil); urea, sodium citrate and sodium azide were of analysis-grade and were from Merck (Darmstadt, Germany). Stock solutions of polymers in water (w/w) were prepared as follows: PEGs = 50%, dextran 2 000 000 = 20%, dextran 505 000, 73 000 and 60 000–90 000 = 40%.

2.2. Screening of reagents and phase diagram determination

A screening of reagents was conducted for phase formation in the presence of polysaccharides. The phase diagrams were constructed according to the procedure developed by Albertsson and Tjerneld [15], by visual inspection of the transition points from the biphasic to the monophasic state for each ATPS. The separation of phases was better observed after centrifugation of the tubes for 2 min at 500 *g*.

2.3. HPSEC analysis

The molecular mass distribution of the polymers was determined by HPSEC using a Waters chromatograph Model 4000 with a controller, an integrator, a Bio-Rad SEC-40XL column (300 × 7.8 mm I.D.) and a differential refractometer electronic unit Model R401 detector at 16× attenuation. Elution was conducted with a 0.02% NaN_3 solution at a flow-rate of 0.9 ml/min. Samples containing 50 μl of each polysaccharide solution (0.4%) were injected. A standard curve was obtained using solutions of dextrans of different molecular masses. The polysaccharides were incubated in the presence of a 7.0 *M* urea solution at 22°C and also at 55°C and in the presence of 1.4 *M* NaCl and sodium citrate solution (22°C), in order to investigate any effect that the salts might have on their elution from the HPSEC column.

3. Results and discussion

For our studies on the formation of ATPS by the legume seed polysaccharides, the synthetic polymer PEG and the microbial dextran of different molecular weights were selected for the investigation of polymer–polymer systems and sodium citrate was selected for the formation of polymer–salt systems.

The yields of the polysaccharide investigated (expressed as the percentage of the dry weight of the seed) were previously characterised [2] and are as follows: 33% for *Schizolobium parahybum*, 42% for *Dimorphandra mollis*, 40% for *Copaifera langsdorffi* and 33% for *Hymenaea corbaryl.*

The molecular mass distributions of the polysaccharides were estimated by HPSEC and a broad dispersion of molecular mass was observed within each fraction. The difference in retention times of the components of the *Dimorphandra mollis* galactomannan fractions, G3 and G4, is shown in the HPLC chromatograms (Fig. 2a and Fig. 2b). The lowest molecular mass found was for the *Dimorphandra mollis* galactomannan (G4, <100 000), which was extracted with hot water. It is possible that some hydrolysis of the polysaccharide might have occurred during extraction, since a large difference was found between the average molecular masses of fractions G4 and G3 (Table 1). The latter was extracted mechanically. The other galactomannan fractions [G2 (*Schizolobium parahybum*) and G3] showed apparent molecular masses of 2 000 000. This agrees with the value of the molecular mass of the galactomannan of locust bean gum, as estimated by Garnier et al. [24] using intrinsic viscosity measurements. According to Dea and Morrison [25] the determination of the molecular masses of galactomannans and other polysaccharides is technically difficult, due to the high viscosities of even dilute solutions and also to polydispersity.

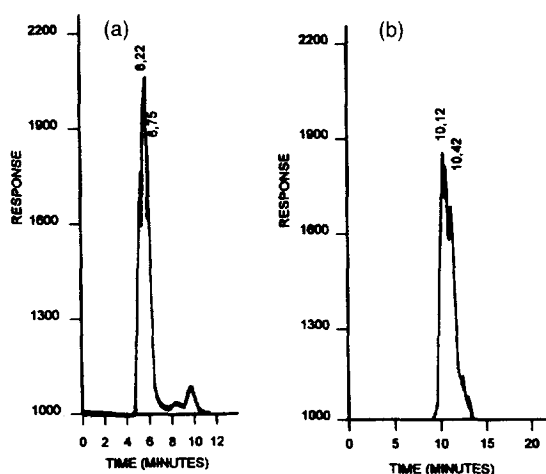

Fig. 2. HPSEC chromatogram of *Dimorphandra mollis* galactomannan on a Bio-Rad SEC-40 XL column, 300 × 7.8 mm I.D., eluted with 0.02% NaN_3 solution at a flow-rate of 0.9 ml/min; IR detector. (a) G3 fraction and (b) G4 fraction.

The phase diagrams of *Dimorphandra mollis* (G3) with dextran 2 000 000 and 505 000 and *Schizolobium parahybum* (G2) with dextran 2 000 000 are shown in Fig. 3 and Fig. 4. The bottom phases of the systems were dextran-rich and of lower viscosity than the top phases. Higher concentrations of polysaccharides were necessary for phase separation with dextran 505 000 than with dextran 2 000 000. The curves show that approximately the same concentrations of both legume seed polysaccharides form ATPS with dextran 2 000 000.

A wide distribution of xyloglucan molecular masses (280 000 to 2 000 000) was found for the *Copaifera langsdorffii* and *Hymenaea courbaril* polymers, the peaks corresponding to a molecular mass of 2 000 000 having the largest area. Particularly strong gels were formed when 1% solutions of xyloglucan of both species were present in 30% sodium citrate solutions after incubation for 24 h; however, at lower concentrations of xyloglucan, phase formation was observed (Table 2).

Relatively lower molecular mass with a large dispersion (100 000–700 000) was observed for the galactan from *Lupinus angustifolius* cotyledons, and

Table 1
Distribution of the apparent molecular mass of polysaccharides from leguminous seeds, determined by HPSEC analysis (SEC XL40)

Fraction	Apparent molecular mass
G1	700 000
G2	2 000 000
G3	2 000 000
G4	<100 000
X1	2 000 000
X2	2 000 000

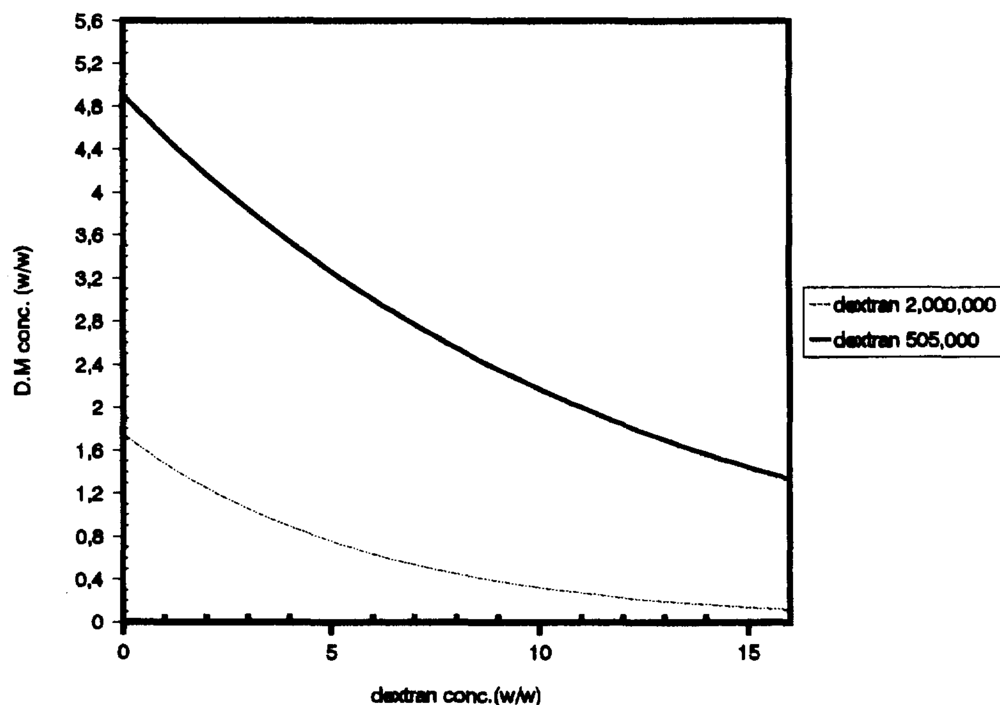

Fig. 3. Binodal curve for *Dimorphandra mollis* (G3 fraction) × dextran 2 000 000 and dextran 500 000.

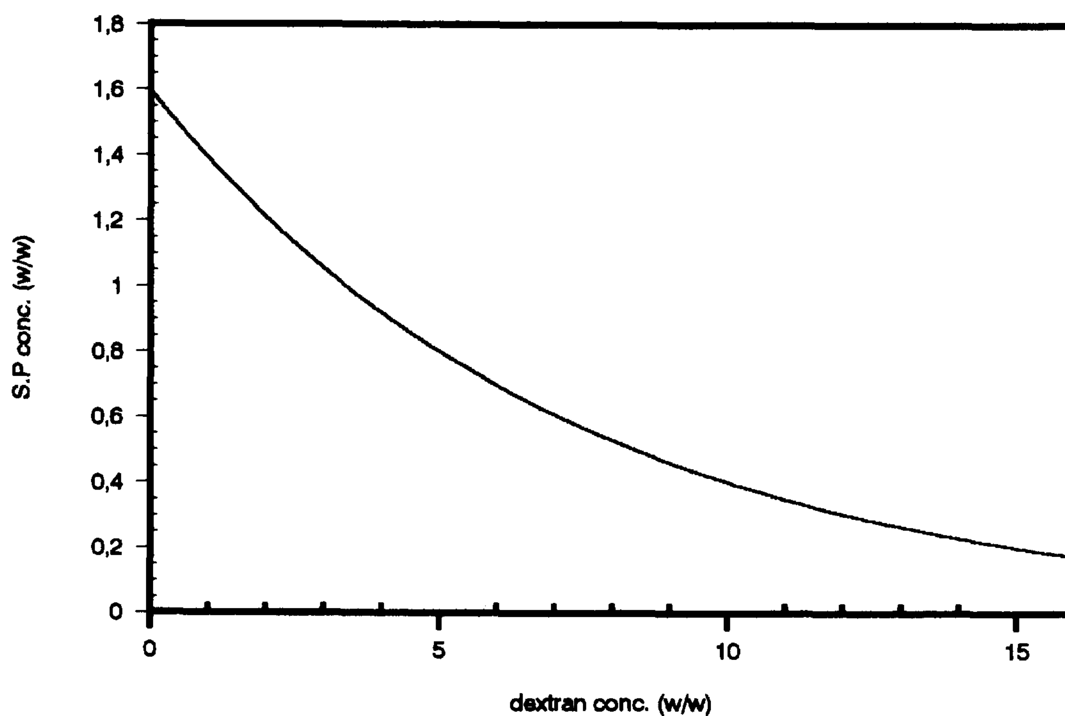

Fig. 4. Binodal curve for *Schizolobium parahybum* (G1 fraction) × dextran 2 000 00.

Table 2
Screening of compounds for the formation of aqueous two-phase systems with solutions of 1% polysaccharides from legume seeds

Reagent	G1	G2	G3	G4	X1	X2
20% Dextran 2×10^6	1 ph.	2 ph.	2 ph.	2 ph.	1 ph.	2 ph.
20% Dextran 5×10^5	1 ph.	2 ph.	2ph.	1 ph.	1 ph.	1 ph.
13% PEG 1500	1 ph.	2 ph.	2 ph.	1 ph.	2 ph.	2 ph.
13% PEG 4000	2 ph.	2 ph.	2 ph.	1 ph.	2 ph.	2 ph.
13% PEG 6000	2 ph.	2 ph.	2 ph.	2 ph.	2 ph.	2 ph.
13% PEG 8000	2 ph.	2 ph.	2 ph.	2 ph.	2 ph.	2 ph.
30% Sodium citrate	liquid	2 ph. (gel)	2 ph. (gel)	1 ph.(gel)	strong gel	strong gel
<30% Sodium citrate	liquid	1 ph.	1 ph.	1 ph.(gel)	2 ph.	2 ph.

ph. = phase

the highest area was found for peaks correponding to a molecular mass of 700 000. Galactan does not form phases with dextrans of different molecular masses and does not form a gel in the presence of sodium citrate, at the concentrations studied.

Four different treatments were used to break a possible aggregation of the polysaccharide chains. These were a 7 *M* urea solution (at room temperature and at 55°C) and NaCl and sodium citrate solutions (at 22°C). No effect on the elution of the polymers from the HPSEC column was observed at either temperature for the urea treatment. However, increasing the concentration of NaCl and sodium citrate, significantly reduced the X1 and X2 retention times (from 6.5 to 6.1 min), suggesting that these salts might have an effect on polysaccharide dispersion.

In the presence of 13% solutions of PEG 1500, 4000, 6000 and 8000, the galactomannans and the xyloglucans apparently formed two phases (polysaccharide in the bottom phase and PEG in the top phase); and, at higher concentrations of PEG, complete precipitation was observed.

4. Conclusions

From the results presented in this work, it is clear that the polysaccharides from legume seeds can promote phase formation in the presence of dextrans, PEG and sodium citrate. On HPSEC analysis, galactomannans and xyloglucans displayed high average molecular masses with a high level of dispersion. They form gels with high concentrations of sodium citrate. These properties could be useful for the application of CWSP as a tool for the purification of biomolecules, such as polysaccharide-degrading enzymes, as well as for studies of the interaction mechanisms of the plant cell wall polymers. The mechanism of affinity partitioning, which uses a combination of the natural ligands present in the polysaccharide surface and controlled conditions in the systems, is suggested for the purification and recovery of polysaccharide-degrading enzymes in ATPS.

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