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BIOLOGICAL ACTIVITIES OF SOME GALACTOMANNANS AND THEIR SULFATED DERIVATIVES

M. MAGDEL-DIN HUSSEIN*, WAFAA A. HELMY and H. M. SALEM†

Department of Natural Products, National Research Centre, Dokki, Cairo; † Department of Biochemistry, Faculty of Agriculture, Cairo University, Egypt

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Key Word Index—Leguminous seeds; *Leucaena* sp.; *Medicago sativa*; lucerne; *Glycine max*; soy bean; *Phoenix dactylifera*; date palm; galactomannan depolymerase; anticoagulation; heparin; fibrinolysis; pentosan sulfuric polyester (Hemoclar); sulfated polysaccharides.

Abstract—Three galactomannans were isolated by extraction, with boiling water; of the whole seeds of *Leucaena* sp. and *Medicago sativa* and the seed hulls of *Glycine max*. A fourth galactomannan was obtained by alkaline extraction of *Phoenix dactylifera* seeds. Chromatographic examination of acid hydrolysates of the four galactomannan preparations revealed the presence of sugar residues other than mannose and galactose. Isolation of galactomannan products, devoid of sugar components other than mannose and galactose, was attempted by applying three fractional precipitation methods. Three such fractions were attained from the gums of *Leucaena* sp. and *M. sativa* (at 40% ethanol) and from that of *G. max* (as its copper complex). An enzyme preparation from germinated seeds of *Leucaena* sp. when incubated with the galactomannan, resulted in a considerable decrease (from 202 to 138) in its degree of polymerization-value and a slight change (from 2.14 to 2.0) in the mannose:galactose ratio. The crude, fractionated and partially degraded galactomannans exhibited considerable anticoagulation and fibrinolytic activities. Sulfation of these polysaccharides improved the biological activities of both the native and enzymatically modified products.

INTRODUCTION

Galactomannans have attracted considerable academic and industrial attention because of their unique chemical and physical properties, in addition to their biological functions. Plant galactomannans are reserve polysaccharides composed of variable proportions of D-mannose and D-galactose residues. The use of these polysaccharides (also known as gums) as substances for mummification can be traced back to 3000 BC in ancient Egypt and, hence, they are often called "Pharaoh's Polysaccharides" [1]. The importance of these polysaccharides can be seen in their wide use in industry, notably in food, pharmaceuticals, cosmetics, paper products, paints, plasters, well-drilling, explosives and fire-fighting.

However, there are few reports on the biological activities of isolated galactomannans. Recently, Liping *et al.* [2] have studied the antiviral activity of a galactomannan isolated from *Pleurotus citrinopileatus*. It exhibited partial anti-Coxsacki B5 activity. Sulfation of this galactomannan led to a change in its conformation, probably due to negative charge exclusion. The sulfated product was shown to have increased anti-virus CB5 activity.

As an additional effort to explore the biological activities of this type of polysaccharides, the present study was directed towards the anticoagulation and fibrinolytic activities of some galactomannan products originating from the seeds of four local plants. Sulfation of these polymers was undertaken in an attempt to improve their biological activities.

RESULTS AND DISCUSSION

Preparation of galactomannans of different molecular characteristics

This was achieved by isolation from different plant sources, by fractional precipitation and by partial enzymatic hydrolysis. Thus, native galactomannans were isolated from the seeds of three leguminous and one non-leguminous plants from local sources. Since the composition of any starting material (e.g., seeds) affects the quality (including the biological activities) and quantity of the final product (e.g., galacto-

* Author to whom correspondence should be addressed.

Table 1. Chemical composition (%) of some galactomannan-containing seeds

Plant source	Ash	Polymeric carbohydrates	LMWC*	Free glucose	Protein	Lipids	Lignin
Leucaena sp.	3.30	43.10	2.10	0.02	36.90	7.20	4.70
Medicago sativa	6.20	39.80	2.80	0.14	43.20	1.65	5.70
Glycine max (hulls)	6.04	57.60	5.60	0.09	15.40	1.67	6.90
Phoenix dactylifera	1.14	54.70	1.20	0.05	16.40	7.40	20.60

* Paper chromatography of low M_r carbohydrates revealed the presence of mannose and galactose (major components) and glucose (minor component).

Table 2. Relative proportions (%) of monosaccharide constituents of seed hydrolysates

Plant source	Galactose	Glucose	Mannose	Arabinose	Xylose	Uronic acid
Leucaena sp.	32.00	31.10	34.50	2.10	—	Traces
Medicago sativa	26.40	34.90	23.90	7.30	—	Traces
Glycine max (hulls)	36.90	40.50	22.60	Traces	—	Traces
Phoenix dactylifera	16.40	27.20	54.60	1.70	Traces	Traces

—, Absent.

mannan), analysis of seeds was done in order to determine to what extent they differed from those used by previous workers. Table 1 shows the chemical composition of the seeds of *Leucaena* sp. *Medicago sativa*, *Phoenix dactylifera* and the seed hulls of *Glycine max*. Table 2 shows the relative proportions of the sugar components of the hydrolysed seeds. The proportions of galactose and mannose are at their highest values in the seed hulls of *G. max* and the seeds of *P. dactylifera*, respectively. Our data are generally in agreement with those published for *Leucaena* sp., [3], *G. max* [4–7] and *P. dactylifera* [8].

Extraction of the three leguminous seeds was achieved with boiling water [9], since they comprise galactomannans of relatively high galactose contents. On the other hand, date-palm seeds were subjected to an alkaline extraction [10], as they are characterized by their higher and lower proportions of mannose and galactose, respectively.

The yields and chemical composition of the isolated galactomannans are recorded in Table 3. The relatively low yields (5.4%) of the seed galactomannan of *M. sativa* is similar to the previously reported value (5.5%) [11], while that of the seed-hulls of *G. max* is higher than the published content (2%) [9]. On the other hand, the galactomannan yields of *Leucaena* sp. and *P. dactylifera* seeds (15.2% and 9.8%) are lower than those reported 20.3% [3] and 22.3% [12], respectively. These differences can probably be attributed to variations in plant origins and extraction conditions.

Chromatographic examination of acid hydrolysates of the isolated gums (Table 4) revealed the presence of major amounts of manose and galactose. Additionally, lesser amounts of other monosaccharides were also recorded in the chromatographed hydrolysates. Among these sugar components, glucose and arabinose were found in all samples, while xylose was detected only in that of *P. dactylifera*. Furthermore, trace amounts of a uronic acid (probably glucuronic acid) were also found in the galactomannan hydrolysate originated from seed hulls of *G. max*. These sugar components may comprise building units of other polysaccharide(s) probably occurring as contaminant(s) in the present galactomannan prep-

Table 3. Galactomannan content of investigated seeds and analytical characters of isolated gums

Plant source	Galactomannan yield (% dry seed)	Total carbohydrates*	Protein*	Ash*
Leucaena sp.	15.20	48.40	11.90	3.10
Medicago sativa	5.40	50.80	30.70	9.10
Glycine max (hulls)	8.30	83.00	5.60	2.10
Phoenix dactylifera†	9.80	53.80	14.40	26.30

* *% of isolated galactomannan.*

†*Extracted with 4% NaOH.*

Table 4. Relative proportions (%) of monosaccharides constituents of galactomannan hydrolysates

Plant origin	Mannose	Galactose	Glucose	Arabinose	Xylose	Uronic acid	M/G ratio
Leucaena sp.	59.00	27.60	12.10	1.10	—	—	2.14
Medicago sativa	49.20	35.40	12.20	3.30	—	—	1.39
Glycine max (hulls)	39.90	31.20	8.10	20.90	—	Traces	1.28
*Phoenix dactylifera**	70.50	11.20	8.20	3.90	6.20	—	6.29

* Extracted with 4% NaOH.
—, Absent.

arations. Calculations of mannose:galactose (M/G) ratios (Table 4) showed significant differences like those previously recorded [1] for the galactomannans of various plant seeds. Generally, the highest and lowest M/G ratios were observed in the gum samples obtained from the seeds of *P. dactylifera* and the seed-hulls of *G. max*, respectively.

Separation of galactomannan products devoid of sugar residues other than mannose and galactose was attempted by using three fractional precipitation methods. These included utilization of ethanol (at various concentrations), copper acetate and barium hydroxide. Using 40% ethanol, two fractions representing the target material were isolated, in two separate experiments, from the seed galactomannans of *Leucaena* sp. and *M. sativa*. A similar fraction was also obtained from the *G. max* galactomannan as its copper complex. Table 5 shows the analytical data for the isolated galactomannan fractions. Generally, all fractions comprised higher proportions of total carbohydrates with lower levels of protein contamination, compared with the corresponding starting materials. Noteworthy, is that the M/G values of the isolated fractions are somewhat lower than those of the corresponding unfractionated materials. This can be attributed to the elimination of some galactomannan chains rich in mannose residues.

In order to prepare another model system of galactomannan chains, the native product from *Leucaena* sp. seeds was subjected to partial hydrolysis by a depolymerase prepared from germinated seeds of the same plant. The reducing power of the digest after 48 h, indicated liberation of reducing sugars equivalent to 26.1% of the starting galactomannan.

The resultant partially hydrolysed gum (P) was then isolated by precipitation with ethanol and comprised ca 76% of the original material. Chromatographic examination of the supernatant revealed the presence of free galactose, mannose and arabinose, in addition to considerable amounts of unidentified oligosaccharides. Complete acid hydrolysis of P, followed by chromatographic analysis, indicated the presence of mannose and galactose in a M/G ratio of 2.0. The degree of polymerization of the native galactomannan and its degraded product (P) was 202 and 138 respectively.

Biological activities

It is well known that some natural polysaccharides and the derivatives of others possess certain biological activities, such as anticoagulation, fibrinolysis, antiviral and antitumor effects. Good examples of such polysaccharides are the anticoagulant herparin, a sulfated polysaccharide from animal origins, and the French fibrinolytic drug, Hemoclar, the trade name of pentosan sulfuric polyester. Our galactomannan samples were examined for their anticoagulation and fibrinolytic activities. The crude galactomannans from *P. dactylifera* and *G. max* exhibited anticoagulation activities comparable to that of standard heparin sodium. However, other two crude products were less active. Also the anticoagulation activities of the three galactomannan fractions isolated from the crude

Table 5. Analytical characters of galactomannan fractions

Fraction	Yield (%)	Total carbohydrates (%)	Protein (%)	M/G-value
F1	61.10	87.20	1.80	1.30
F2	48.80	79.20	3.00	1.00
F3	8.70	96.60	1.10	1.04

F1: Isolated from galactomannan from *Leucaena* sp.
F2: Isolated from galactomannan from *M. sativa*.
F3: Isolated from galactomannan from *G. max*.

Table 6. Fibrinolytic activities of isolated galactomannans and their sulfated derivatives*

Plant origin	Before sulfation	After sulfation
Leucaena sp.	+ +	+ + + +
M. sativa	?	?
G. max	?	?
P. dactylifera	+	+ + +
Modified product (P)	+ + + +	+ + + + +
Standard Hemoclar*	+ + + +	—

* Polysaccharide concentration = 2 mg per tube.
+ + + + + = Lysis of more than 75% of plasma clots.
+ + + + = Lysis of 75% of plasma clots.
+ + + = Lysis of 50% of plasma clots.
+ + = Lysis of 25% of plasma clots.
+ = Lysis of less then 25% of plasma clots.
? = Very weak activity.

products of *Leucaena* sp., *M. sativa* and *G. max* were lower than those of the original materials. Furthermore, partial enzymatic hydrolysis of the native galactomannan isolated from *Leucaena* sp. seeds, did not alter its anticoagulation activity.

Determination of fibrinolytic activities (Table 6) of the gum products towards plasma clots, indicated that the native galactomannans from *Leucaena* sp. seeds showed a fibrinolytic activity equivalent to half that of standard preparation of Hemoclar, while that of *P. dactylifera* seeds gave a quarter value of the standard activity. On the other hand, the products from the seed-hulls of *G. max* and *M. sativa* exhibited only very weak fibrinolytic activities. Again, the isolated galactomannan fractions were less active than the corresponding native materials. The enzymatically modified product (P) from *Leucaena* sp. showed fibrinolytic activity equivalent to that of a standard Hemoclar preparation.

In an attempt to improve the biological activities of the various galactomannan products, they were subjected to a sulfation process and the resultant derivatives re-examined for their anticoagulation and fibrinolytic activities. The anticoagulation activities of the sulfated derivatives from the gums of *M. sativa* and *G. max*, were ca ten times higher than those of the corresponding unsulfated ones. Also, the sulfated product from the gum of *Leucaena* sp. seeds exhibited anticoagulation activity double that of its original galactomannan. Similarly, sulfation of the enzymatically modified product (P) enhanced its anticoagulation activity three-fold. On the other hand, sulfation of the isolated galactomannan fractions had little effect on their anticoagulation activities. Sulfation of the galactomannan from *P. dactylifera* seeds also did not improve its anticoagulation activity.

Determination of the fibrinolytic activities of the modified galactomannans (Table 6) revealed that the sulfated products from the seed gums of *Leucaena* sp. and *P. dactylifera* now had higher activities similar to that of the standard, Hemoclar. The sulfated product of the partially degraded gum (P) also exhibited a fibrinolytic activity higher than that of the standard. Taking into consideration the similarity between the M/G values of the product (P) and its mother galactomannan, one can attribute the improving activities of the former and its sulfated derivative to their relatively lower degree of polymerisation (DP). On the other hand, the sulfated galactomannans from the seeds of *M. sativa* and the seed-hulls of *G. max* showed very low fibrinolytic activities. Again, the sulfated derivatives of the isolated galactomannan fractions showed fibrinolytic activities comparable to those of their corresponding unsulfated products.

The aforementioned results, indicate a relationship between the chemical composition and the biological activities of most of the galactomannans prepared in the present work. In most cases, galactomannans comprising higher proportions of mannose residues are more active than those of lower M/G values. Shortening of the chain-length of such galactomannans led to an improvement in the biological activities of the resulted partially degraded product. Similarly, most of the sulfated galactomannans comprising higher proportions of mannose units exhibited biological activities higher than those of the sulfated derivatives of lower M/G values. Noteworthy is that a galactomannan product with a higher M/G ratio and lower DP value showed the highest fibrinolytic activity recorded in the present study. Accordingly, variation in the biological activities of the different galactomannan models prepared during this work, is probably due to some conformational differences in the molecules of these polysaccharides.

EXPERIMENTAL

Plant material

Whole seeds of *Leucaena* sp., *M. sativa* and *P. dactylifera* were milled prior to extraction. Seeds of *G. max* were crushed and the hulls separated.

Chemicals

The glucose oxidase kit is a commercial preparation (Srtanbio Lab., U.S.A.). Heparin was purchased from Sigma. Hemoclar is a commercial product prepared by Clin-Midy, Paris, and supplied by the Nile Co. for Pharmaceuticals, Cairo, Egypt. Plasma was prepared from a citrated sample of sheep blood purchased from The Egyptian Organization for Biological Products and Vaccine Production.

Analytical methods

Ash content was determined by heating the sample to a constant wt at 800°. Organic N was determined by Kjeldahl's method and multiplied by 6.25. For

H_2O-sol. products, protein determination was carried out by the method of Ref. [13]. Total lipids were isolated by Soxhlet extraction with *n*-hexane and determined according to Ref. [14]. Low M_r carbohydrates (LMWC) were attained by Soxhlet extraction with 85% EtOH for 24 h [15]. The resulting alcoholic extract was decolourized by boiling with charcoal, concd under vacuum at 45° and then examined by PC using *n*-BuOH–Me_2CO–H_2O (4:5:1) [16] and aniline phthalate [17] and aniline xylose [18] as spray reagents. Quantitative determination of LMWC in the decolourized alcoholic extract was achieved by the pHOH–H_2SO_4 method [19] and the quantities were obtained from graphs constructed from measurements on solns containing mannose and galactose (1:1). Specific determination of free glucose was done using a glucose-oxidase kit. Polymeric carbohydrates were determined in the seed materials after complete acid hydrolysis [20] of the samples pre-extracted with 85% EtOH. The resulted acid hydrolysates were examined by PC as described above. Quantitative determination of separated sugars was carried out according to Ref. [21]. Carbohydrate content of the acid hydrolysates were determined by phenol–H_2SO_4 method [19] and the quantities obtained from standard curves constructed from measurements on solutions comprising appropriate sugars in appropriate proportions. For isolated galactomannan products, acid hydrolysis was done according to Ref. [22] followed by quantitative PC [16, 21]. Total carbohydrates were determined in the acid hydrolysates by the phenol–HSO_4 method [19] using appropriate graphs constructed from measurements on solutions containing appropriate proportions of the appropriate sugars. Lignin was determined according to the method of Ref. [23].

Extraction of water-soluble galactomannans

Adopting the method of Ref. [9] with some modifications, the leguminous galactomannans were extracted with hot H_2O. Ground seeds (or seed-hulls) (5 g) were extracted, successively, with H_2O (200 ml × 3) at 100°, under reflux, for 3 h (1 × 3). After filtration, the combined extracts were treated with 3 vols of EtOH and the resulted ppt isolated by centrifugation, dried and weighed.

Extraction of alkali-soluble galactomannan

The H_2O-insol. galactomannan of palm-seeds was isolated by alkaline extraction [10]. Ground seeds (5 g) were successively extracted by boiling under reflux with 4% NaOH (200 ml × 3). After filtration, the resultant extracts were combined, neutralized with HOAc and dialysed against H_2O (48 h). Precipitation of the galactomannan was accelerated by addition of 3 vols of EtOH and the polysaccharide ppt was then isolated by centrifugation, washed with EtOH, dried and weighed.

Fractional precipitation with EtOH

This was achieved by gradual addition of EtOH to a 1% soln of the crude galactomannan prepn. At various EtOH concns (20, 40, 50, 60 and 70%), the resultant precipitated frs were separated by centrifugation.

Fractional precipitation with Cu^{2+}

The galactomannan prepn from the seed-hulls of *G. max* was fractionated as its Cu complexes using the method of Ref. [24].

Attempted fractionation with Ba^{2+} ions

Attempted fractionation of the seed galactomannan of *P. dactylifera* was done according to Ref. [25].

Preparation of sulfated galactromannans

Sulfation of galactomannan was carried out with chlorosulfonic acid [26] and the resultant product was isolated with 3 vols of MeOH. Purification of the sulfated galactomannan was performed by repeated dissolution in H_2O and reprecipitation with MeOH.

Enzymatic hydrolysis of Leucaena *sp. galactomannan*

A galactomannan depolymerase was prepared from germinated seeds of *Leucaena* sp. according to Ref. [27], with slight modifications. The resulted partially purified enzyme was then examined for its protein content [13] and its β-mannanase and α-galactosidase activities by measuring total reducing sugars [28, 29] obtained using a mannan preparation from *Codium dichotomum* [30] and raffinose, respectively. Partial hydrolysis was carried out in a reaction mixt. consisting of 0.5 g of substrate dissolved in 320 ml H_2O and 5 ml of partially purified enzyme preparation (E/S: 1/43.5) with few drops of toluene. After incubation at 37° for 48 h, total reducing sugars were determined [28, 29] and 3 vols of EtOH were then added. The resultant ppt was isolated by centrifugation, dried and weighed. The supernatant and an acid hydrolysate of the ppt were examined separately by PC for their sugar constituents.

Determination of degree of polymerization

The DP of native and enzymatically modified galactomannan of *Leucaena* sp. seeds were determined according to Ref. [31].

Determination of sulfate hemi-ester groups

Purified sulfated galactomannan products were hydrolysed with HCl [32] and the liberated sulfate ions assayed turbidmetrically [33]. Found: SO_4^{2-}%: 59.4, 47.8, 43.9, 42.4 and 45.1 for the sulfated gal-

actomannans from *M. sativa*, *G. max*, *Leucaena* sp., *P. dactylifera* and the enzymatically modified product (P), respectively.

Biological activities

Anticoagulation activities of the various polysaccharide preparations were determined according to the USP [34] and compared with that of a standard heparin sodium preparation. Fibrinolytic activities were assayed as lysis-percentages of the plasma clots when incubated at 37° with the investigated polysaccharides. Preparation of plasma clots was achieved under the same conditions previously applied in the blank-tests throughout the determination of anticoagulation activities [34]. For comparison, a standard preparation of pentosan sulfuric polyester sodium (Hemoclar) was used as a control.

REFERENCES

1. Dea, C. I. M. and Morrison, A., *Advances in Carbohydrate Chemistry and Biochemistry*, 1975, **31**, 241.
2. Liping, Z., Yishen, Z., Fei, S. and Zhongyan, L., *Shengwu Huaxue Yu Shengwu Wuli Xuebao*, 1994, **26**, 417, in *CA*, 1995, **122**, 259168p.
3. Arora, S. K. and Hissar, U. N. J., *Starch/Starke*, 1985, **37**, 109.
4. Aspinall, G. O., Begbie, B. and Mckay, J. E., *Cereal Science Today*, 1967, **260**, 233.
5. Kawamura, S., *Technical Bulletin of Faculty of Agriculture. Kagawa University*, 1967, **15**, 117.
6. Nelson, G. A., Talley, L. E. and Aronovsky, S. I., *Cereal Chemistry*, 1950, **8**, 59.
7. Smith, A. K. and Circle, S. J., *Soybeans: Chemistry and Technology*, Vol. 1, *Proteins*. AVI Publishing Co. Inc. Westport, Connecticut, 1978, pp. 61–92.
8. Takashi, M. and Yachiyo, M., *Nippon Nogel Kagaku Kaishi*, 1958, **23**, 829, in *CA*, 1959, **53**, 7328.
9. Whistler, R. L. and Saarino, J., *Journal of the American Chemical Society*, 1957, **79**, 6055.
10. Jindal, V. K. and Mukherjee, S., *Indian Journal of Chemistry*, 1970, **8**, 417.
11. Andrews, P., Hough, L. and Jones, J. K. N., *Journal of American Chemical Society*, 1952, **74**, 4029.
12. Jindal, V. K. and Mukherjee, S., *Current Science*, 1969, **38**, 459.
13. Lowry, O. H., Rosenbrough, N. J., Farr, A. L. and Randall, R. J., *Journal of Biological Chemistry*, 1952, **193**, 265.
14. *Official Methods of Analysis of Association of Official Agricultural Chemists*, 11th edn. Washington, DC, 1970.
15. Abdel-Fattah, A. F. and Hussein, M. M., *Phytochemistry*, 1970, **9**, 721.
16. Jaume, G. and Knolle, H., *Angewandte Chemie*, 1956, **68**, 243.
17. Partridge, S. M., *Nature*, 1949, **164**, 443.
18. Saarino, J., Nikasuari, E. and Gustafsson, C., *Suomen Kemistilehti B*, **25B**, 25.
19. Dubois, M., Gills, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F., *Analytical Chemistry*, 1956, **28**, 350.
20. Fischer, F. G and Dörfel, H., *Hoppe-Seyler's Zeitschrift für Physiologische Chemie*, 1955, **302**, 186.
21. Wilson, C. M., *Analytical Chemistry*, 1959, **31**, 1199.
22. Haug, A. and Larsen, B., *Acta Chimica Scandinavica B*, 1962, **16**, 1908.
23. Adams, G. A., *Methods in Carbohydrate Chemistry*, Vol V, Ed. R. L. Whistler, and M. L. BeMiller. Academic Press, New York, 1965, p. 185.
24. Aspinall, G. O. and Whyte, J. N. C., *Journal of Chemical Society*, 1964, 5058.
25. Timell, T. E., *Svensk Papperstidning*, 1961, **64**, 651.
26. Hussein, M. M., *Egyptian Patents*, 1994, No. 19381 August, CO8G63100, A61K9100.
27. Hyline, J. W. and Sawai, K., *Journal of Biological Chemistry*, 1964, **239**, 990.
28. Nelson, N., *Journal of Biological Chemistry*, 1944, **153**, 375.
29. Somogyi, M., *Journal of Biological Chemistry*, 1952, **195**, 19.
30. Love, J. and Percival, E., *Journal of Chemical Society*, 1964, 3338.
31. Hay, G. W., Lewis, B. A., Smith, F. and Unrau, A. M., in *Methods in Carbohydrate Chemistry*, Vol V. ed. R. L. Whistler and M. L. BeMiller. Academic Press, New York, 1965, p. 251.
32. Larsen, B., Haug, A. and Painter, J. T., *Acta Chimica Scandinavica B*, 1966, **20**, 219.
33. Garrido, M. L., *Analyst*, 1964, **89**, 61.
34. *The United States Pharmacopeia*. Mack Publishing Company, 1960, p. 317.