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Polysaccharides from *Melittis melissophyllum* L. herb and callus

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Dedicated to Prof. S. Kohlmünzer[1] on the occasion of his 80th birthday

For comparison of the water-soluble polysaccharides from *Melittis melissophyllum* L. herb with that produced by Melittis callus cultures the polymeric carbohydrates were extracted from both sources, fractionated by IEC and GPC and the respective fractions analysed concerning sugar composition and linkage characteristics. The dominant structures found in all fractions isolated from herb material and callus were type-II arabinogalactans with a $(1 \rightarrow 3)$-galactose backbone and arabinose-galactose side chains. No significant differences were found between herb and callus polysaccharides. To optimize the *Melittis* cell culture systems the culture media were varried systematically. A modified Murashige-Skoog (MS) medium, supplemented with GA_3, NAA and BAP was found to be most suitable for large-scale production of callus material.

1. Introduction

Melittis melissophyllum L. (Lamiaceae) is a perennial plant growing in shady separate places of leafy forests in Europe [1]. Herba Melittidis has wide applications in folk medicine as a sedative, antispasmodic, antiphlogistic, diuretic and antiulcer remedy [2, 3] and is also monographed in the French Pharmacopoeia [4]. The chemical composition of *Melittis melissophyllum* is only partly analysed until now [5–11]. The presence of phenolic acids such a *p*-hydroxy-benzoic, vanillic, syringic, *p*-coumaric, *o*-coumaric, caffeic, ferulic and melilotic acids and a variety of flavones with luteolin and luteolin 7-O-glucoside as main compounds have been demonstrated [5]. Additionally the occurrence of coumarin and the iridoid glucosides melitoside, monomelitoside, harpagide, acetyl-harpagide, ajugol and ajugoside have been detected in the plant material [6–10]. The essential oil which accounts for about 0.2% consists of mainly chrysanthenyl acetate, a-terpineol and caryophyllene oxide [11]. Despite the fact that the herb has quite a high carbohydrate content, which gets obvious during aqueous maceration of the herb, leading to highly viscous extracts, no literature is available on the nature of the carbohydrate-pool in the plant. On the other side it is known that *in vitro* cultured cells and calli derived from higher plants are capable to synthesise higher amounts of polysaccharides either as cell-bound secondary polymers or as extracellular polymers [12–14].

The aim of the following investigation was the isolation and fractionation of *Melittis melissophyllum* L. polysaccharides from the plant material as well as from callus material in order to study the phytochemical characteristics and potential similarities between the polymer structures from these different sources.

2. Investigations and results

2.1. Fractionation and structural features of polysaccharides from Melittis herb

A crude, slightly brownish polysaccharide fraction (CPS) was obtained from an aqueous extract of *Melittis* herb after precipitation of high-molecular weight products with ethanol and subsequent lyophilisation in a 1.1% yield. CPS were fractionated by ion-exchange chromatography on DEAE-Sephacel using a step gradient system into one neutral (I) and four acidic fractions (II to V) (Fig.). GPC on Superose 12 indicated great heterogeneity of these fractions that were further fractionated on semipreparative scale on Sephacryl S-300 into 11 subfractions (Table 1). The average molecular weights were less than 56 kDa, which is quite low molecular for water-soluble plant polysaccharides.

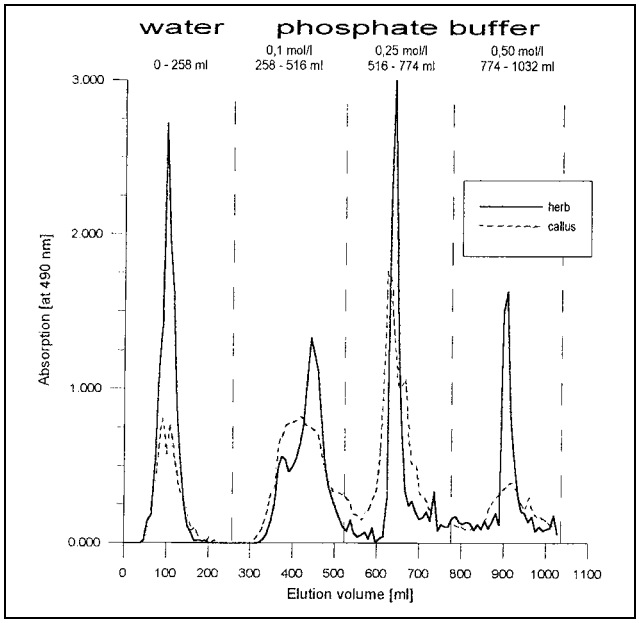

Fig.: Elution profile of crude polysaccharides (CPS) from *Melittis melissophyllum* L. herb and callus after ion exchange chromatography on DEAE–Sephacel

For structure elucidation, the polysaccharides from subfractions, occurring in relevant yields, were hydrolysed and the respective alditol acetates of neutral sugars were analysed (GLC) yielding arabinose and galactose as main constituents (Table 2). Fractions eluted from IEC at higher buffer concentrations had higher rhamnose (3 to 47%) and uronic acid contents (3 to 20 %).

Methylation analysis of representative neutral polysaccharides and carboxyl-reduced acidic polymers (Table 3) showed all fractions to consist of principally similar arabinogalactans with a 1,3-/1,3,6-galactan backbone and side chains with different fine structures, characterised mainly by branched rhamnose residues and different amounts of 1,4-galacturonic acid. The amount of some sugars especially rhamnose, determined by GLC of alditol acetates and by methylation analysis of the carboxyl-reduced poly-

Table 1: Comparative fractionation scheme for polysaccharides from *Melittis melissophyllum* L. herb and callus

***Melittis melissophyllum* L. herb** Crude polysaccharides (CPS) – yield 1.1%					***Melittis melissophyllum* L. callus** Crude polysaccharides (CPS) – yield 2.0%			
ION-EXCHANGE CHROMATOGRAPHY (DEAE-SEPHACEL)								
Neutral fraction	0.1 mol/l buffer eluate	0.1 mol/l buffer eluate	0.25 mol/l buffer eluate	0.5 mol/l buffer eluate	Neutral fractions	0.1 mol/l buffer eluate	0.25 mol/l buffer eluates	0.5 mol/l buffer eluates
9%	3%	19%	17%	8%	7 and 8%	13%	7 and 4%	6 and 2%
I	II	III	IV	V	C. I and C. II	C. III	C. IV and C. V	C. VI and C.VII

GEL PERMEATION CHROMATOGRAPHY

Ia	Ib	Ic	IIa	IIb	IIIa	IIIb	IVa	Va	Vb minor amount	Vc minor amount	C.Ia	C.Ib	C.IIa	C.IIb	C.III	C.IV	C.V	C.VI	C.VII minor amount
51 kDa	15 kDa	10 kDa	37 kDa	7 kDa	46 kDa	6 kDa	56 kDa	56 kDa	16 kDa	9 kDa	11 kDa	4 kDa	9 kDa	4 kDa	46 kDa	46 kDa	46 kDa	46 kDa	46 kDa

Table 2: Monosaccharide composition of the polysaccharide fractions from *Melittis melissophyllum* L. herb as determined by GLC. Values indicate the respective molar composition

Fraction	Ia	Ib	Ic	IIa	IIb	IIIa	IIIb	IVa	Va	Vc
Sugar component										
Rhamnose						3	5	2	11	47
Arabinose	33	32	28	26	32	24	17	10	13	12
Xylose							1		2	
Mannose					tr.*		6			
Galactose	67	68	72	71	65	68	63	75	62	21
Glucose			tr.*				3		3	
Uronic acid				3	3	5	5	13	10	20

* tr.: traces

Table 3: Methylation analysis of neutral polysaccharide fractions (Ia to Ic) and of selected carboxyl-reduced acidic polysaccharides from *Melittis melissophyllum* L. herb. Values indicate the respective molar composition

Sugar component	Linkage type	Ia	Ib	Ic	IIa	IIIb	IVa	Va
Galactose	1–			6				
	1,3–	7	3	8	8	6	11	15
	1,4–	5		7	2	18		
	1,6–	11	18	19	6	13	7	6
	1,3,6–	47	48	30	60	35	57	42
Arabinose	1–	20	18	18	6	10	4	8
	1,3–	1	4	4	1	1	2	2
	1,2–	9	9	8	4	4		
	1,3,5–				4	2	3	3
Xylose	1,2–							1
Rhamnose	1–				1	2		4
	1,2–				5	1	2	7
Glucose	1,4,6							
Galacturonic acid*	1–					6		
	1,4–				3		14	12

* determined as deuterated galactose residues

mers was slightly different for some fraction. Therefore it is likely that rhamnose is directly linked to the galacturonic acid residues, which causes a pronounced stabilising effect of this aldobiuronic acid against hydrolysis; cleavage of this glycosidic bond occurs only under drastic conditions or in the carboxyl-reduced polysaccharide.

These data are consistent with polysaccharides consisting of a 1,3-linked galactan backbone with a high degree of branching of the galactose residues via position 6 with side chains of 1-arabinose, arabinose-(1 → 3)-arabinose, rhamnose-(1 → 4)-galacturonic acid-(1 → 6)-galactose or rhamnose-(1 → 4)-galacturonic acid. The structures of such acidic arabinogalactans are consistent with similar type II arabinogalactans from higher plants with a β-linked galactose backbone, α-configurated arabinose side-chains and terminal α-rhamnose residues. The same anomeric properties are therefore supposed also for the *Melittis* polymers. We were not able to perform sufficient precise ^{13}C NMR data in order to prove such a configuration precisely because of the low yields of polysaccharides during fractionation.

2.2. Callus cultures

Parts of seedlings (shoot tops, joints, parts of leaves, rootlets and little stems) were placed on MS agar medium with addition of various growth regulators, especially with the auxin 2,4-D in different concentrations (Table 4). Light-green or light-yellow callus was formed from all explants on most variants of media. Optimal growth and callus-formation was obtained from parts of little stems and rootlets on media enriched with 2,4-D and benzylaminopurine (variant C) or 2,4-D and kinetin (variant E). Other media compositions were shown not so much effective as variants C and E.

For obtaining solid callus tissue was transferred on the same media. Tissue obtained under these conditions showed very slow growth. In order to speed up the potential growth different variants of MS medium were tested (Table 4, H, K–P) and the respective values of growth index (relative change of fresh mass) were compared. As a result we found a MS medium named variant P supplemented with BAP (1 mg/l), NAA (0.5 mg/l) and GA_3 (0.25 mg/l) to be optimal for effective callus production with an excellent growth factor of about 18. The resulting callus tissue obtained after 1 to 1.5 months was dark green, solid and ready for further multiplication.

Table 4: Callus cultures of *Melittis melissophyllum L.*

Explant	Results	Variant	MS medium supplemented with plant growth regulators (mg/l)						(g/l)		Growth index I_G *
			GA_3	NAA	Kin	BAP	2,4–D	IAA	m-inosit	casein	
Seeds	Seedlings	A	0.5		0.25						n.d.
Parts of plantlets	Formation of callus	B			1		0.25				n.d.
		C				1	0.25				n.d.
		D					0.5				n.d.
		E			1		0.5				n.d.
		F					1				n.d.
		G			1		1				n.d.
		H			0.1		1				n.d.
		I				1		1			n.d.
		J		1		1					n.d.
Callus tissue	Growth of callus	B–J									n.d.
		H			0.1		1				3.9 ± 0.6
		K			0.5		1				4.2 ± 0.8
		L			0.5		1		0.5	0.5	4.2 ± 0.8
		M			0.5		1		0.5		4.5 ± 0.7
		N			0.5		1			0.5	4.6 ± 0.7
		O				1	0.5				4.1 ± 0.7
		P	0.25	0.5		1					18.2 ± 2.4

* average growth index as mean of 5 independent series from each 20 individual cultures (BAP: 6-benzylaminopurine; Kin: kinetin; 2,4-D: 2,4-dichlorophenoxyacetic acid; m-inosit: mesoinositol; GA_3: gibberellic acid; NAA: a-naphtaleneacetic acid; IAA: indole-3-acetic acid)

2.3. Fractionation and structural features of polysaccharides from Melittis callus, variant P

In order to compare polymeric carbohydrates produced by *Melittis* callus with that from the plant material callus polysaccharides were isolated and fractionated by the same extraction and chromatographic procedures as used above. The profile of fractions and subfractions was comparable to that obtained from the herbal material (Fig., Table 1). Also determination of sugar composition revealed the presence of type II arabinogalactans which was proven by acetylation and methylation analysis (Tables 5, 6). The main structural features found in all fractions analysed were polymers with $(1 \rightarrow 3)$-galactose backbone and a high degree of branching via position 6. A slightly different structure was found for the polymer in fraction C.VI, a galactan with about 40% 1,6- and 30% 1,3,6-galactose residues. In all other polymers studies the amount of 1,6-galactose units was found to be less. This means that the majority of the callus polysaccharides are polymers with a galactan backbone and mixed arabinose-galactose side chains while the polymer from C.VI seems to be a $(1 \rightarrow 3)$-galactan with $(1 \rightarrow 6)$-galactose side chains and only little amounts of other carbohydrates.

Table 5: Monosaccharide composition of the polysaccharide fractions from *Melittis melisophyllum* L. callus as determined by GLC. Values indicate the respective molar composition

Sugar component	C.Ib	C.IIa	C.III	C.IV	C.V	C.VI
Rhamnose			4	5	5	4
Arabinose	16	49	30	32	18	8
Galactose	84	41	60	51	68	79
Uronic acid			6	12	9	9

* tr. = traces

3. Discussion

Within the investigations on polysaccharide content and structures from *Melittis* plant material and callus, the dominance of strongly heterogenic arabinogalactans was shown for both systems. The main structural features of polymers from herb and callus are comparable, indicating that the cell culture system is not introducing a different pathway for the production of polysaccharides. In addition, the amounts of polymeric carbohydrates found in both systems were comparable. Similar investigations on mucilage production from plant material and the respective cell cultures revealed the presence of nearly identical xyloglucans from tobacco leaves and cultures [13] in contrast to results found with polymers from *Echinacea purpurea* – glucuronoxylans in herbal material [15] and arabinogalactans in suspension cultures [16] or *Plantago lanceolata*, which produced different polysaccharides in

Table 6: Methylation analysis of neutral polysaccharide fractions (C.Ib and C.IIa) and of selected carboxyl-reduced acidic polysaccharides from *Melittis melissophyllum* L. callus. Values indicate the respective molar composition

Sugar component	Linkage type	C.Ib	C.IIa	C.III	C.IV	C.V	C.VI
Galactose	1–	35		17	15		1
	1,3–	8	10	10	9	19	
	1,4–	16	2				
	1,6–		5	2	2	4	44
	1,3,6–	18	29	27	23	43	27
	1,4,6–	8		2	2	2	5
Arabinose	1–	10	23	22	26	15	3
	1,3–		9	3	4	2	3
	1,2–	4	3				
	1,3,5–	1	18	3	2	1	
Xylose	1,2–						
Rhamnose	1–			4			
	1,2,4–				5	4	4
Glucose	1,4,6						
Galacturonic acid*	1–						
	1,4–			10	12	10	13

* determined as deuterated galactose residues

herbal material and cell cultures [17]. The triggering into polysaccharide biosynthesis pathways in cell culture may thereby be induced by the composition of the respective media. Therefore, it may be worth for future investigations to isolate and characterise carbohydrates in *Melittis* cell cultures that have only a weak growth rate, indicating sub-optimal conditions.

4. Experimental

4.1. Materials

Melittis melissophyllum L. herb was collected in the forest near Lublin (Poland) and air dried. A voucher specimen is deposited at the Department of Pharmaceutical Botany, Collegium Medicum, Jagiellonian University, Krakow (Poland) under the reference number MM1a.
All chemicals used were of analytical grade quality, purchased from Sigma (Steinheim, Germany) or Merck (Darmstadt, Germany). DEAE-Sephacel and Sephacryl S-300 were purchased from Pharmacia (Uppsala, Sweden).

4.2. General methods

Total carbohydrates were assayed with the phenol-sulphuric acid test [18]. Determination of uronic acids was performed using *m*-hydroxydiphenyl [19] and galacturonic acid as reference material. Carbohydrate-containing solutions were concentrated under reduced pressure at temperatures below 40 °C. Cellulose membranes with MWCO 3500 Da were used for dialysis. The molecular weight distribution of polysaccharides was determinated by GPC using standard dextrans (Sigma, Steinheim, Germany). Polysaccharides were hydrolysed with trifluoroacetic acid 2 mol/l at 121 °C for 60 min. Alditol acetates of neutral sugars were prepared according to Blakeney et al. [20]. GLC was performed on a Hewlett-Packard HP 6890 system with mass selective detector at 220 °C with helium as carrier gas (pressure 1.3 bar) on an HP-225 fused silica capillary column (30 m × 0.25 mm i.d., film thickness 0.25 μm).
Reduction of acidic polysaccharides to the carboxyl-reduced polymers was accomplished in the presence of carbodiimide and $NaBD_4$ as described by Taylor and Conrad [21]. Methylation analysis was performed according to the Hakamori method, modified by Harris [22].

4.3. Callus cultures

In vitro cultures were started from *Melittis* seeds received from various European botanical gardens. The preparation of seeds, their sterilisation and conditions of the cultures growth were performed according to Skrzypczak and Skrzypczak [23]. Seedlings obtained on MS medium [24] supplemented with GA_3 and kinetin (Table 4) were used as material to start callus cultures.
Seven variants of MS medium (H, K-P) were prepared. There were 20 Erlenmeyer flasks for each variant. 0.50 g of callus tissue was transferred to each flask. Growth lasted 30 days and later the callus tissue was collected and weighed. The growth index (I_G) was calculated for each variant of MS medium. Such series of callus growth were repeated in five independent series and average growth index (I_G) was calculated for each medium.

$$I_G = \frac{W_F - W_I}{W_I}$$

I_G: growth index; W_I: initial fresh weight of callus tissue (g); W_F: final fresh weight of callus tissue (g)

4.4. Isolation and fractionation of polysaccharides from Melittis herb

Dried and milled herb (500 g) was pre-extracted with ethanol at room temperature and then twice with water for 24 h at 35 °C. The water extract was concentrated and poured dropwise into ethanol 96% (V/V) to achieve a final ethanol concentration of 70% (V/V). To complete the precipitation of ethanol-insoluble material the mixture was kept for 48 h at 4 °C and was subsequently centrifuged for 15 min at 4500 rpm. The pellet (crude polysaccharides CPS) was lyophilised.

Ion exchange chromatography of CPS was performed on a DEAE-Sephacel column (27 × 2.7 cm) equilibrated with sodium phosphate buffer. The column was eluted with water at a flow rate of 0.4 ml/min to yield the neutral fraction and then with a stepwise gradient using sodium phosphate buffer, pH 6.0, with increasing ion strength from 0.1 to 1.0 mol/l. Carbohydrate-containing fractions were pooled, concentrated, dialysed, and lyophilised. Gel-permeation chromatography (GPC) was performed on calibrated Superose™ 12 FPLC column and on Sephacryl S-300 column (78 × 1.7 cm) with sodium chloride (0.1 and 0.15 mol/l, respectively) as mobile phase and fraction sizes of 0.5 and 2 ml, respectively. Carbohydrate-containing fractions were pooled, concentrated, dialysed and lyophilised.

4.5. Isolation and fractionation of polysaccharides from Melittis callus

The isolation procedure in principle was similar to that performed for isolation of polysaccharides from *Melittis* herb. There were 84 g of dried and milled callus as starting material. GPC of callus polysaccharides was performed on Sephacryl S-300 column (78 × 1,7 cm) with sodium chloride 0.15 mol/l as mobile phase and fraction sizes of 2 ml.

Acknowledgements: Financial support of this study was obtained by the Fonds der Chemischen Industrie by a grant to A. H. The authors thank Mrs. K. Meier for valuable technical assistance.

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Received February 9, 2000
Accepted March 8, 2000

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