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GLYCOPROTEINS OF THE OPIUM POPPY

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Key Word Index—*Papaver somniferum*; Papaveraceae; opium poppy; glycoprotein; cell-wall protein; serine-*O*-galactoside.

Abstract—Two carbohydrate–protein fractions were isolated from the water-soluble biopolymer from opium poppy capsules by chromatography on SP-Sephadex. The carbohydrate chains are composed of arabinose, rhamnose, xylose, mannose, glucose, galactose, galacturonic acid, glucuronic acid and 4-*O*-methyl glucuronic acid. Methylation analysis indicated a high degree of branching suggesting a very complex structure. Treatment of the glycoprotein with NaOH in the presence of $NaBH_4$ resulted in a significant decrease in the serine and threonine content. The carbohydrate side chains released contained the sugar alcohol, galactitol. These results indicate that polysaccharide chains are linked to protein via serine-*O*-galactoside linkages.

INTRODUCTION

The capsule of the opium poppy contains a pectin-type polysaccharide [1, 2]. When this polysaccharide was subjected to partial hydrolysis, several acidic and a few neutral oligosaccharides were obtained [3, 4]. The pectin-type polysaccharide gives viscous aqueous solutions, causing difficulties in the manufacturing of morphine and other opium alkaloids from the capsules. It is therefore important from a scientific, as well as from a practical, point of view to undertake a study of this water-soluble polysaccharide of the opium poppy capsule. The material appears to be a glycoprotein—as has been found for other carbohydrate polymers usually classified as polysaccharides [5, 6].

RESULTS AND DISCUSSION

Aqueous extraction of powdered poppy capsules pretreated with organic solvents gave, after dialysis and freeze-drying, a carbohydrate-containing polymer in 4% yield (raw material). This polymer was separated into two fractions by chromatography on SP-Sephadex C-25 (Fig. 1). Fraction A was eluted with 0.1 M acetic acid and fraction B with 0.1 M Na-acetate buffer pH 5. The yield of A after repeated evaporations to remove acetic acid and lyophilization was 75% and of B after dialysis and lyophilization was 3.5% of raw material.

Both fractions contained protein and carbohydrate. Fraction B contained more protein than fraction A relative to carbohydrate (Fig. 1). As the polymer seems to contain bound morphine and codeine [7] a SP-Sephadex column was used to separate the polymer-containing alkaloids from free alkaloids. Results concerning the alkaloid content will not be reported in this paper.

The MWs of A and B were estimated on a Sepharose 4B column. The column was calibrated against dextrans and proteins of known MW. As proteins and carbohydrates of the same MW are eluted differently, owing to their different shapes, the MWs are only approximate.

Table 1 gives the composition of total carbohydrate, protein and uronic acid estimated by colorimetric methods. Because there are substantial proportions of neutral monosaccharides in the glycoprotein fractions, the carbazol reaction gives ambiguous results [8].

The composition of component sugars after methanolysis (Table 1) shows low yields compared with the total carbohydrate content of the fraction. This can be caused by incomplete methanolysis of the uronic acid residues. According to the results from the method of Bitter and Muir [9] a major part of the uronic acid content is lost during methanolysis. Chambers and Clamp [10] propose that it is sufficient to methanolyse with 1 M HCl/MeOH for all glycoproteins. Methanolysis with 4 M or 6 M HCl will give much degradation and unknown peaks in the chromatogram. This is perhaps not true for all glycoproteins. In *Opuntia ficus-indica* Miller [11] 6 M HCl in MeOH had to be used for some fractions to achieve complete methanolysis.

The component sugars in fraction A were further identified as the corresponding alditol acetates. Fraction A was carboxyl-reduced twice (with EDC/$NaBD_4$), hydrolysed (2 N H_2SO_4), reduced and acetylated. The carboxyl reduction was carried out with $NaBD_4$ to distinguish alditols originating from aldoses from those originating from hexuronic acids. The alditol acetates were analysed by GC–MS (3% OV-225 column) and the following acetylated sugar alcohols identified: rhamnitol, arabinitol, traces of fucitol, xylitol, 4-*O*-meglucitol*, galactitol/galactitol*,

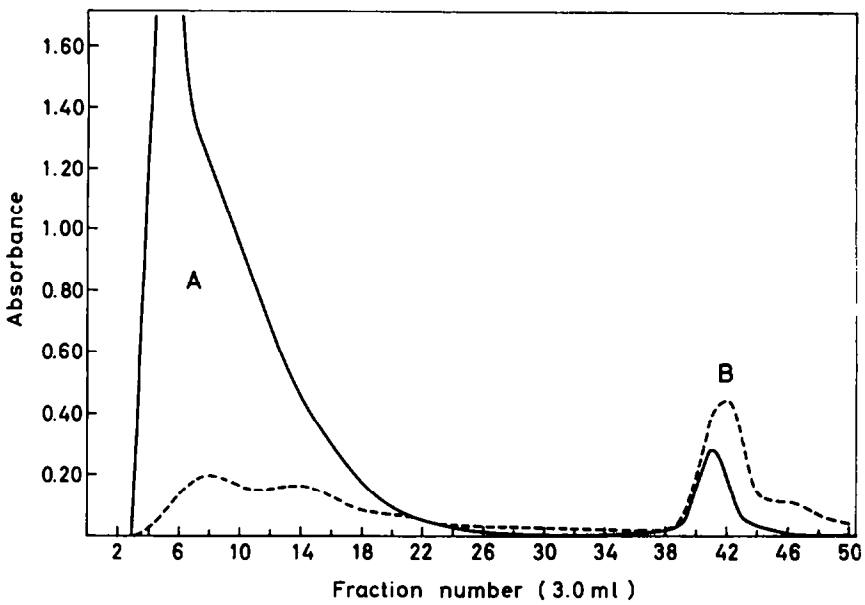

Fig. 1. Elution of *P. somniferum* raw extract on SP-Sephadex in H^+ form. The column (27 × 2.0 cm) was eluted with 0.1 M acetic acid. At fraction 26 the eluting agent was changed to 0.1 M sodium acetate buffer pH 5.0. ———, carbohydrate; —— protein.

glucitol/glucitol*. The asterisk indicates alditols originating from the corresponding hexuronic acid.

The present polymer appeared to be of both carbohydrate and protein character. The commonly detected glycopeptide bond in plant glycoproteins, the hydroxyproline-*O*-arabinoside bond, is of minor importance in this biopolymer in view of the trace amounts of hydroxyproline present (Table 2). The existence of a serine/threonine-*O*-galactoside bond has recently been demonstrated in glycoproteins from *Cannabis sativa* [12], potato lectin [13] and *Urtica dioica* [6]. An *O*-glycosidic linkage between a sugar and the hydroxyl group of peptide-linked serine and threonine will be labile to alkali, resulting in β-elimination of the carbohydrate moiety. Treatment of fraction B with NaOH–$NaBH_4$ gave a decrease in serine and threonine of 12 and 13%, respectively, and an increase of 20% in alanine. The other amino acids showed minor differences. Only one sugar alcohol, galactitol, was detected after the reductive base catalysed hydrolysis of the glycoprotein. This indicates that galactose was the only sugar involved in linking carbohydrate to serine/threonine in the polypeptide chain.

The unsuccessful methylation of the native polymer A may be ascribed to the high content of uronic acid [14]. For this reason, and also because the MS fragmentation pattern of partly methylated hexuronic acid acetates has not been fully elucidated, the uronic acid units in the glycoprotein were reduced to the corresponding neutral sugar residues. The product obtained after carbodiimide activation and $NaBD_4$ reduction was easier to methylate by the Hakomori procedure.

By the carbodiimide activation/$NaBD_4$ reduction procedure the carbohydrate–peptide bond will be broken and degradation of the carbohydrate chain can be avoided.

After hydrolysis and conversion of the partly methylated monosaccharides into the corresponding alditol acetates, the mixture was analysed by GC–MS. The results are given in Tables 3 and 4 and indicate the presence of previously identified products. Fraction B

Table 1. MW and composition of fractions A and B

		A	B
MW range		17 000–45 000	5000–26 000
Weight % composition	Total carbohydrate	73.2	46.9
	Protein	4.7	84.9
	Uronic acid	62.4	18.6
	Arabinose	5.8	6.2
	Rhamnose	4.5	1.5
	Xylose	6.4	2.3
	Mannose	2.6	1.9
	Glucose	4.9	8.0
	Galactose	9.0	7.2
	Galacturonic acid	18.6	7.5
	Glucuronic acid	0.4	0.5

Table 2. Amino acids in fraction B

Component	B (mol %)
Asp	13.5
Thr	6.9
Ser	7.8
Glu	12.3
Pro	5.6
Gly	12.1
Ala	8.1
Val	8.3
Met	0.6
Ile	3.9
Leu	5.3
Tyr	2.5
Phe	3.8
His	1.1
Lys	5.4
Arg	3.1
Hyp	tr.

having less uronic acid was methylated directly without any preceding carboxyl reduction.

Both fractions contain arabinofuranose and -pyranose end groups. Fractions A and B have glucose/mannose end groups, A in addition has galactose end groups. Galacturonic/glucuronic acid end groups are also present in A. Fraction A, being carboxyl-reduced, appeared to have $1\rightarrow3$ bound glucuronic acid and $1\rightarrow4$ bound glucuronic and galacturonic acid. $1\rightarrow2$, $1\rightarrow4$ bound galacturonic and traces of $1\rightarrow2$, $1\rightarrow4$ bound glucuronic acid are also present in the chain. The hexoses are located both as branch points, unsubstituted in the chain, and as end groups. This is probably the case also for rhamnose and arabinose.

The IR spectrum of fraction A showed an intense absorption band at 1735 and 1250 cm^{-1} suggesting the presence of ester linkages [15]. Fraction B showed only a shoulder at these absorption bands.

Table 5 gives the content of *O*-acetyl groups and total ester in A and B. In fraction A a major part of ester groups are due to acetylated primary alcohol groups, in B most of the ester groups originate from esterified uronic acid.

The present results indicate that the water-soluble biopolymers A and B are appreciably different. They are probably both cell wall constituents though they are hydroxyproline-poor glycoproteins. Brown and Kimmins [16], however, have obtained hydroxyproline-poor glycoproteins from *Phaseolus vulgaris* after virus inoculation or wounding.

EXPERIMENTAL

Materials. Papaver somniferum, of Turkish origin, was grown in the Botanical Garden at the University of Oslo.

Table 3. Previously identified, partially methylated alditol acetates obtained from fraction A, reduced with EDC–$NaBD_4$ prior to methylation

R_{TMG}	Primary MS fragments (*m/e*)	Alditol acetate
0.45	45,117,131,161,175	2,3,5-Tri-*O*-methylarabinitol
		2,3,4-Tri-*O*-methylrhamnitol
0.59	117,161	2,3,4-Tri-*O*-methylarabinitol/xylitol
0.88	45,117,131,161,189,233	3,5-Di-*O*-methylarabinitol
		2,4-Di-*O*-methylrhamnitol
1.03	45,47,117,161,163,205,207	2,3,4,6-Tetra-*O*-methylglucitol/mannitol/glucitol*
1.10	117,189	2,3-Di-*O*-methylarabinitol
		3,4-Di-*O*-methylarabinitol
1.21	45,47,117,161,163,205,207	2,3,4,6-Tetra-*O*-methylgalactitol/galactitol*
1.55	117,131,261	2-*O*-Methylrhamnitol
		4-*O*-Methylrhamnitol
1.75	189,203	3-*O*-Methylrhamnitol
1.99	45,47,117,161,189,233,235	2,4,6-Tri-*O*-methylglucitol/glucitol*
		3,4,6-Tri-*O*-methylglucitol/mannitol
2.10	45,47,117,161,189,233,235	2,3,6-Tri-*O*-methylgalactitol/galactitol*
		2,3,4-Tri-*O*-methylmannitol
2.27	45,47,117,161,189,233,235	2,3,6-Tri-*O*-methylglucitol/glucitol*
		2,3,4-Tri-*O*-methylglucitol
2.72	117,161,189	2,3,4-Tri-*O*-methylgalactitol
3.34	45,117,261	2,6-Di-*O*-methylgalactitol
3.68	45,47,117,189,233	3,6-Di-*O*-methylgalactitol*
3.97	45,(47),117,189	3,6-Di-*O*-methylglucitol*
4.91	117,261	2,3-Di-*O*-methylhexitol
5.31	117,189,233	2,4-Di-*O*-methylgalactitol
6.51	117	2-*O*-Methylglucitol
7.53	118	3- or 4-*O*-methylglucitol

R_{TMG}: R_t relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol.
* Originating from corresponding hexuronic acid.

Table 4. Partially methylated alditol acetates obtained from fraction B

R_{TMG}	Primary MS fragments (m/e)	Alditol acetate
0.44	45, 117, 131, 161, 175	2,3,5-Tri-O-methylarabinitol
		2,3,4-Tri-O-methylrhamnitol
0.57	117, 161	2,3,4-Tri-O-methylarabinitol
0.85	45, 117, 131, 161, 189, 233	3,5-Di-O-methylarabinitol
		2,4-Di-O-methylrhamnitol
		3,4-Di-O-methylrhamnitol
1.00	45, 117, 161, 205	2,3,4,6-Tetra-O-methylglucitol/mannitol
1.06	117, 189	2,3-Di-O-methylarabinitol
		3,4-Di-O-methylarabinitol
1.40	117	2-O-Methylrhamnitol
2.21	117, 161, 189, 233	2,3,4-Tri-O-methylglucitol/mannitol

R_{TMG}: R_t relative to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylglucitol.

Table 5. Esterification in fraction A and B (μmol/mg)

	A	B
O-Acetyl groups (15)	1.90	0.32
Total ester groups (22)	2.30	1.45

Powdered capsules without seeds were subjected to successive Soxhlet-extraction with petrol (bp 60–80°), $CHCl_3$ and MeOH to remove non-polar substances.

Aqueous extraction and purification. The pretreated material was extracted by H_2O at 60°, dialysed and freeze-dried. The lyophilized polymer amounted to 4% yield. The polymer was dissolved in 0.1 M HOAc and applied to a column (30 × 3 cm) of SP-Sephadex C-25 (H^+ form). The column was eluted with 0.1 M HOAc until no more carbohydrate was eluted. Subsequently the column was eluted with 0.1 M NaOAc buffer pH 5. (Fig. 1.) The fractions (3 ml) were tested for carbohydrate and protein and the carbohydrate/protein positive material of fraction A was isolated after repeated evapns and freeze-drying. The carbohydrate/protein positive material corresponding to fraction B was freed from NaOAc by dialysis followed by concn and freeze-drying.

General methods. Concns were carried out under red. pres. below 50° on a rotary evaporator. Dialysis was performed with magnetic stirring against H_2O; toluene was added to prevent microbial growth. Amino acids were analysed on an amino acid analyser after hydrolysis of the sample (10 mg) with M HCl for 20 hr at 110° under N_2. Carbohydrate was determined by the PhOH–H_2SO_4 method [17] with a mixture of galacturonic acid, galactose, arabinose, glucose and rhamnose (12:2:2:1:1) as standard. Uronic acid was estimated with the carbazol method as modified in ref. [9] with α-D-galacturonic acid as standard. Protein was determined by the method of ref. [18] with human albumin as standard. IR spectra were recorded for samples (1–2 mg) in KBr discs.

All GLC analyses were performed on a FID instrument using N_2 as carrier gas. For GC–MS the column was coupled to a Varian CH 7 low-resolution mass spectrometer.

Methanolysis. The dried polymer (2–3 mg) was heated with M HCl in dry MeOH in a sealed tube for 20 hr at 80°, mannitol was used as int. standard. Trimethylsilylation of the resulting Me glycosides was performed with Py, hexamethyldisilazane and chlorotrimethylsilane (5:2:1) according to ref. [19]. The products were analysed by GLC on a 3% SE-52 column (390 × 0.2 cm) on Varaport 30. The temp. programme started at 140° with an increase of 1°/min for 10 min, followed by an increase of 2°/min to 170°, and finally 4°/min to 225°.

Gel filtration. MWs were determined by gel filtration on a column of Sepharose 4B (35 × 2.5 cm). The column was calibrated against the Dextran T series from Pharmacia as well as against different proteins. Eluting agent was 25 mM Tris–HCl buffer 7.2; fractions of 3 ml were collected.

Carboxyl-reduction of polymer. The sample was treated with 225 mg EDC (1-ethyl-3(3-dimethylaminopropyl)-carbodiimide) in 10 ml H_2O at pH 4.75 for 2.5 hr at room temp. according to ref. [20]. Subsequently 410 mg $NaBD_4$ were added and the reduction conducted for 2 hr at room temp. $NaBD_4$ was decomposed by addition of 2 M HCl and the soln dialysed overnight before evapn to dryness. To obtain complete reduction the procedure was carried out twice.

Methylation. The sample (15 mg) in dry DMSO was methylated by the Hakomori methylation procedure [21] using 2 M methylsulfinyl carbanion and MeI. The methylated polymer was hydrolysed with 90% HCOOH (4 hr at 100°, followed by 1 hr at 100° after addition of the equal vol. of H_2O) and the alditol acetates prepared. The partially methylated alditol acetates were extracted with $CHCl_3$ and analysed by GC–MS on a 3% OV-225 column (200 × 0.25 cm) at 180°.

Estimation of O-acetyl groups was carried out by the method of ref. [15]. The products were analysed by GLC on a column (400 × 0.2 cm) of 10% SP-1200/1% H_3PO_4 on Chromosorb WAW at 80° with HOPr as int. standard.

Total ester content. Uronic acid esters and acetylated hydroxyl groups were estimated colorimetrically with hydroxamine–$FeCl_3$ by the method of ref. [22]; EtOAc was used as standard.

Alkaline degradation in the presence of $NaBH_4$. The sample (40 mg) dissolved in M $NaBH_4$–0.1 M NaOH was kept at 50° for 6 hr. Excess $NaBH_4$ was decomposed with 2 M HCl and boric acid removed as Me borate by repeated evapns of MeOH. The resulting material was desalted by chromatography on a BioGel P-10 column (100–200 mesh) with 10% EtOH, 3 ml per fraction. The low and high MW fractions were collected and freeze-dried. Part of the low MW fraction was hydrolysed with 6 M HCl and analysed for amino acids. Another part of the same fraction was methanolysed, TMSi-derivatized and analysed by GLC.

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