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THIN-LAYER CHROMATOGRAPHIC ANALYSIS OF ENZYMIC HYDROLYSATES OF AGAR

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SUMMARY

The most suitable solvent systems for the analysis, by cellulose thin-layer chromatography of the neutral and charged oligosaccharides formed by the enzymic hydrolysis of agar have been evaluated. As the mixture of oligosaccharides is not adequately resolved by one or two dimensional thin-layer chromatography to enable identification of the individual components, the neutral and charged sugars are first separated from each other on DEAE-Sephadex A-25 (Cl^-). The solvent system for the optimum separation of the neutral sugars depends on the degree of polymerisation of the oligosaccharides under investigation. A good separation of the charged oligosaccharides is obtained in the solvent system butan-1-ol–acetic acid–water (4:1:2).

INTRODUCTION

Agar is a family of polysaccharides obtained from the agarophytes of the Rhodophyceae (red seaweeds). The agarose component of agar is essentially neutral and has been shown to have a structure in which 3-linked β-D-galactopyranose residues and 4-linked 3,6-anhydro-α-L-galactopyranose residues alternate[1]. This repeating sequence is masked in agaropectin, the charged polysaccharide complex of agar. D-Galactose residues can be replaced by the pyruvic acid ketal, 4,6-O-(1′-carboxyethylidene)-D-galactose[2] and the L-galactose residues can be replaced with sulphated galactoses[3]. D-Glucuronic acid has also been reported to be present[4]. In both agarose and agaropectin some of the D-galactose units can be replaced with 6-O-methyl-D-galactose[5]. The extent of masking varies from species to species.

All known enzyme systems which degrade agar do so by cleaving the β-1,4-linkage between the D-galactose and 3,6-anhydro-L-galactose residues[6–8]. The basic neutral unit formed is the disaccharide neoagarobiose[6], the other neutral oligosaccharides formed are multiples of this. The R_{Gal} values of these oligosaccharides as reported by various workers[6–8] together with the R_{Gal} values of the di- and tetrasaccharide in which one of the D-galactose units is replaced by 6-O-methyl-D-galactose[9] are shown in Table I. The enzymic hydrolysate of agar also contains charged oligosaccharides which so far have not been adequately resolved and identified.

TABLE I

R_{Gal} VALUES FOR NEUTRAL OLIGOSACCHARIDES OBTAINED BY ENZYMOLYSIS OF AGAR

Method used. (I) Ascending paper chromatography in solvent system D[6], (II) Descending paper chromatography in solvent system E[7], (III) Cellulose TLC in solvent system E (double developed)[8,9].

Oligosaccharide	*R_{Gal} values*		
	I	*II*	*III*
6^1-O-Methyl-neoagarobiose	—	—	1.70
Neoagarobiose	1.30	1.35	1.30
6^3-O-Methyl-neoagarotetraose	—	—	1.05
Neoagarotetraose	0.62	0.75	0.80
Neoagarohexaose	0.34	0.32	0.46
Neoagaro-octaose	0.15	0.12	0.25

In an investigation into the mode of action of bacterial agarases we needed to develop solvent systems which would separate neutral oligosaccharides with a wide range of D.P.*. A chromatographic method of separating the charged oligosaccharides had also to be found.

EXPERIMENTAL

Materials and methods

Cellulose layer. Microcystalline cellulose (Camag D.S.O., 15 g in 80 ml of distilled water) was layered to a thickness of 250 μ on glass plates and then air dried.

Indicator reagent. The modified naphthoresorcinol reagent[7] was used. This is the most sensitive indicator for oligosaccharides containing 3,6-anhydro-L-galactose. The spray reagent consists of two parts ethanolic sulphuric acid (375 ml of ethanol plus 100 ml of concentrated sulphuric acid) and one part naphthoresorcinol solution (0.2% in ethanol). No heat was applied to the plate after spraying as this tends to char the cellulose. The oligosaccharide spots are more distinct under UV light.

Solvents. All solvents used were reagent grade obtained from Fisher Ltd. The solvent systems used for cellulose TLC analysis during the course of this investigation were as follows: (A) Butan-1-ol–ethanol–water (3:1:1); (B) Butan-1-ol–ethanol–water (3:2:2); (C) Butan-1-ol–ethanol–water (1:1:1); (D) Butan-1-ol–acetic acid–water (4:1:2); (E) Butan-1-ol–pyridine–water (2:1:1). Solvent system D must be made up daily in order to obtain reproducible results.

Column chromatography. DEAE-Sephadex A-25 (Pharmacia) was prepared in the chloride form by washing successively with hydrochloric acid (0.5 *N*), sodium hydroxide (0.5 *N*), hydrochloric acid (0.5 *N*) and then extensively with distilled water.

Agar. The following agars were used: (a) Difco Bacto Agar; (b) *Gelidium cartilagineum* agar.

Preparation of agaropectin components. Previous studies[10] have shown that successive extraction of the commercial agar at room temperature and at 50° yields two charged polysaccharide complexes. The charged polysaccharides in each eluant being obtained free of neutral agarose by precipitation with cetyl pyridinium chloride.

* D.P. = degree of polymerisation

In the text these polysaccharides will be referred to as agaropectin (20°) and agaropectin (50°).

Enzyme. The purified extracellular agarase from *Pseudomonas atlantica*[11] was used to hydrolyse the polysaccharides.

RESULTS AND DISCUSSION

Neutral oligosaccharides

In this study we found that solvent system B has several advantages over solvent system E which has been used previously for the separation of neutral oligosaccharides obtained by the enzymic hydrolysis of agarose. Only one development is necessary and the sensitivity with the naphthoresorcinol spray reagent under UV light is markedly increased as there is no background absorption due to pyridine. This is important when monitoring an enzymic reaction in which the quantity of an intermediate might be very small. The R_{Gal} values for the oligosaccharides with D.P.'s between 2 and 8 are shown in Table II.

Solvent system A gives an excellent separation of the di- and tetrasaccharides but was unsuitable for higher oligosaccharides (Table II). This was therefore the solvent of choice for the preparative TLC of these two oligosaccharides.

TABLE II

COMPARISON OF THE R_{Gal} VALUES OF NEUTRAL OLIGOSACCHARIDES ON CELLULOSE TLC USING DIFFERENT SOLVENT SYSTEMS

Oligosaccharide	*D.P* [a]	*Solvent A*	*Solvent B*	*Solvent C*
Neoagarobiose	2	1.30	1.11	—
Neoagarotetraose	4	0.51	0.87	—
Neoagarohexaose	6	0.19	0.65	—
Neoagaro-octaose	8	0.05	0.41	0.57
	10	—	—	0.42
	12	—	—	0.30
	14	—	—	0.18

[a] D.P. = degree of polymerisation.

Neutral oligosaccharides with D.P.'s 8 to 14 are well separated in solvent system C (Table II). Multiple developments in this solvent made preparative cellulose TLC possible for these higher oligosaccharides. Fig. 1 compares the separation of the neutral oligosaccharides in solvents A, B and C.

These solvent systems have been found to be extremely valuable in monitoring how various agarases degrade neutral high molecular weight oligosaccharides which have been prepared by the partial enzymolysis of agarose, and purified by preparative thin-layer chromatography using the most suitable solvent system.

Charged oligosaccharides

The mixture of neutral and charged sugars obtained by the enzymic hydrolysis of complete agar and various agaropectin fractions is not resolved by one dimensional TLC as the charged oligosaccharides have the same R_{Gal} values as the neutral oligo-

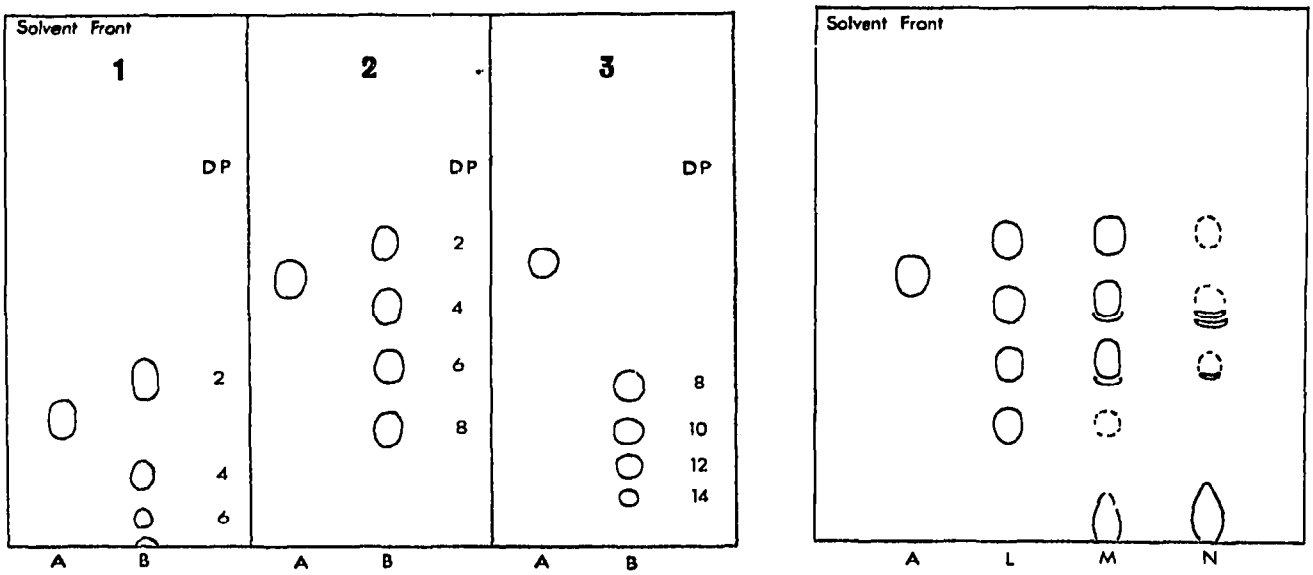


Fig. 1. Separation on cellulose TLC of the neutral oligosaccharides formed by the enzymic degradation of *Gelidium cartilagineum* agar. A comparison of the separation of various D P ranges in different solvents. A in each case is galactose and B the neutral oligosaccharides. Plate 1 was developed in solvent system A, plate 2 in solvent system B and plate 3 in solvent system C.

Fig. 2. Comparison using solvent system B of the enzymic hydrolysate of agarose (L), agaropectin (50°) (M) and agaropectin (20°) (N). A is the reference sugar galactose.

saccharides in some solvents and in others the charged sugars run as poorly defined spots. Fig. 2 compares the enzymic hydrolysates of two agaropectin fractions and the hydrolysate of agarose using solvent B. The "crescents" due to the charged sugars in a neutral solvent can be reduced by using the basic solvent E but the effect is not eliminated.

The mixture of neutral and charged sugars can be partially resolved by two dimensional TLC. The homologous series of neutral oligosaccharides lie along the diagonal and are well separated from the charged sugars. This procedure is not useful in characterising the charged oligosaccharides, which lie off the diagonal, as they often streak and their absolute positions are difficult to reproduce (Fig. 3).

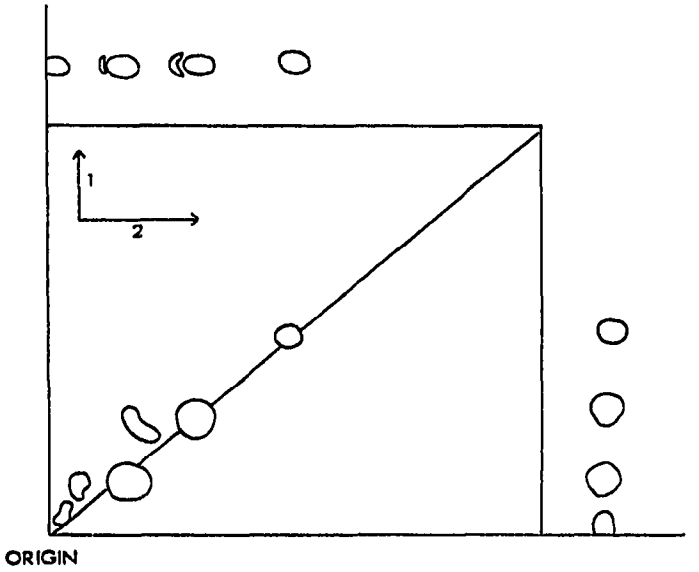
TABLE III

COMPARISON OF THE R_{Gal} VALUES OF NEUTRAL AND CHARGED OLIGOSACCHARIDES USING SOLVENT D

Neutral oligosaccharides D.P.	R_{Gal}	*Charged oligosaccharide**	R_{Gal}
2	1.10	a	0.91
4	0.80	b	0.64
6	0.51	c	0.38
8	0.32	d	0.23
10	0.18		

* a, b, c, d refers to oligosaccharides of unknown structure which contain a pyruvate group as the only charged group. The designation a, b, c, d is also used in Fig. 4.

A good separation of the charged oligosaccharides on cellulose TLC was only obtained after completely separating them from the neutral oligosaccharides. A convenient method of carrying out this separation was by column chromatography on DEAE-Sephadex A-25 (Cl^-). The neutral oligosaccharides are eluted from the gel with distilled water and the charged oligosaccharides with a solution of sodium chloride (2.0 *M*). The charged oligosaccharides were desalted on Sephadex G-25 before being examined by cellulose TLC.



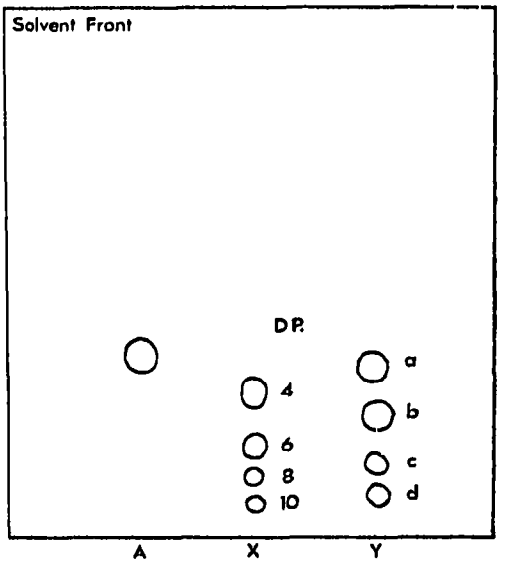


Fig. 3. Two dimensional separation of the complete hydrolysate of Difco Bacto Agar. Direction 1 solvent system E and direction 2 solvent system B.

Fig. 4. Comparison of the separation in solvent D of the charged oligosaccharides (Y) and the neutral oligosaccharides (X) formed by the enzymic degradation of *Gelidium cartilagineum* agar or Difco Bacto Agar. A is the reference sugar galactose.

Poor resolution of the charged oligosaccharides from Difco Bacto Agar and *Gelidium cartilagineum* agar was obtained in neutral and basic solvents, but an excellent separation was obtained in solvent system D (Fig. 4). The R_{Gal} values of the principle charged sugars are shown in Table III. Chemical analysis of these oligosaccharides have indicated that they contain a combined pyruvic acid molecule as the only charged group. The sulphated oligosaccharides formed by the enzymolysis of agar are of high molecular weight presumably because the agarose cannot cleave a β-1,4-linkage close to a sulphated galactose unit[12].

In solvent system D the neutral oligosaccharides have similar R_{Gal} values to the charged oligosaccharides, Table III, and hence it is not possible to study the complete enzymic hydrolysate of agar in this solvent as the higher yield of neutral oligosaccharides tends to mask the presence of the charged oligosaccharides.

CONCLUSION

The most suitable solvent system for the separation of the neutral series of oligosaccharides depends on their D.P. For the di- and tetrasaccharide the best

solvent is solvent A. Oligosaccharides with a D.P. between 2 and 8 are separated in solvent B and the range of D.P. from 8 to 14 in solvent C.

The charged oligosaccharides can only be examined after prior separation from the neutrals on DEAE-Sephadex. Solvent D gives a good separation of the charged oligosaccharides containing a pyruvic acid molecule. The sulphated oligosaccharides are of higher molecular weight and to date no solvent system is known which adequately separates them.

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REFERENCES

1 C. ARAKI, *Bull. Chem. Soc. Japan*, 29 (1956) 543.
2 S. HIRASE, *Bull. Chem. Soc. Japan*, 30 (1957) 68.
3 C. ARAKI, *Proc. 5th Intern. Seaweed Symposium*, Pergamon Press, London, 1966, p. 3
4 C. ARAKI, *J. Chem. Soc. Japan*, 59 (1937) 1214.
5 S. HIRASE AND C ARAKI, *Bull. Chem. Soc Japan*, 34 (1961) 1048
6 C. ARAKI AND K. ARAI, *Bull Chem. Soc Japan*, 29 (1956) 339
7 W. YAPHE, *Can. J. Microbiol*, 3 (1957) 987.
8 M. DUCKWORTH AND J. R. TURVEY, *Biochem. J.*, 113 (1969) 687
9 J. R. TURVEY AND J. CHRISTISON, *Biochem. J.*, 105 (1967) 317
10 W YAPHE, *Proc. 5th Intern. Seaweed Symposium*, Pergamon Press, London, 1966, p 333.
11 W. YAPHE, L. MYCHAJLOWSKA AND K. C. HONG, *Bact. Proc.*, (1968) 24.
12 M. DUCKWORTH AND J. R. TURVEY, *Biochem. J.*, 113 (1969) 693.