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(Arrived: January 14, 1967)

A new Purity Criterion for α and β Cyclodextrin Using Partition Chromatography on Cellulose Columns

By J. N. J. J. LAMMERS, Eindhoven (Netherlands)

Introduction

Cyclodextrins (Schardinger dextrins, cycloamyloses, CD) are cyclic polysaccharides consisting of α-1,4 bound glucose units. α CD consists of six of these units and β CD of seven.

The preparation of the CD mixture is simple because separation from its by-products can be accomplished by selective precipitation. However, separation and purification of α and β CD is much more difficult. Therefore, the most common impurity in α CD is β CD and vice versa. With respect to this, satisfactory purity criteria for the individual CD are lacking in literature.

The specific optical rotation of solutions in water is frequently used as such (1, 2), but the method is not very sensitive to the presence of small amounts of impurities: an amount of 3 % (w/w) of one CD in the other cannot be detected.

The solubility is not a good criterion because of the wide variety of values found in literature for α and for β CD (1–6), owing to the fact that more than one hydrate modification of α CD (7, 8, 9) seems to exist. Moreover the authors do not mention the water content of the samples investigated.

The paperchromatographic method (9, 10) and the TLC method (11) cannot be used because the detection is not sensitive enough (9).

The form of the crystals, when CD crystallises from a 60 % (v/v) 1-propanol in water solution, is characteristic of α and β CD (1) but not sufficiently sensitive to the presence of impurities.

A more precise criterion for the purity of α and β CD has been developed using partition chromatography on cellulose columns. The method is in principle the same as the one reported by PULLEY and FRENCH (12) but is modified in order to be able to detect small amounts.

Experimental

Materials

Cyclodextrins: are prepared according to the method of FRENCH et al (13). Their purification is described by WIEDENHOF and LAMMERS (9).

Cellulose: Whatman Standard Grade Cellulose powder.

1-butanol: 98–100 % Riedel – De Haën A. G. Seelze, Hannover (Germany).

Ethanol: Chemically pure 96 %. Koninklijke Nederlandse Gist- en Spiritusfabriek, Delft (Netherlands).

Water: Redistilled and boiled to remove air.

Preparation of the Column

Cellulose powder is suspended in a mixture of 1-butanol/ethanol[1])/water (42 : 29 : 29 % by volume). This solvent mixture has the same composition as the eluting solvent. The suspension is deaerated in a vacuum desiccator. The composition of the solvent will change during this operation, but this change can be disregarded because before the column is used it is irrigated with fresh solvent.

The cellulose column is prepared in an all-glass tube having an inside diameter of about 3 cm and a length of 65 cm. In order to maintain a constant temperature of 25 °C the tube is provided with a jacket connected with a thermostat. The lower part of the tube is provided with a stopcock. Some glasswool is placed in the bottom end of the tube and a 1 cm thick layer of white sand (50–70 mesh) is poured on to it. Now some water is poured into the column and the sand is stirred with a glass rod in order to clear it from air bubbles. A little of a Celite[2]) suspension in water, deaerated in the same way as the cellulose suspension, is introduced into the tube to the height of 1 cm. The water is then allowed to drain off by opening the stopcock until its level reaches the surface of the celite. The stopcock is now closed. The tube is half filled with solvent, cellulose suspension is added, and the cellulose is allowed to settle slowly. Then the stopcock is opened again, the pressure below the stopcock being reduced to about 0.6 atm. The solvent is sucked off and more suspension

[1]) Calculated on a basis of 100 % ethanol.

[2]) Celite no. 503 (100—140 mesh), Johns Manville Corp., New York (USA).

is slowly and continuously added until the cellulose surface reaches the desired height. By passing more solvent through the cellulose column the bed will compress. Flushing with 0.5–1 l solvent during 1–2 hours is continued until no further compression is observed and the filtrate is perfectly clear. Before using the column it is tested with a mixture of thymol blue and crystal violet — dissolved in ethanol — to observe the separation characteristics (14).

Protection of the Column

It is important to keep the column free from air bubbles and to prevent it from running dry. Therefore the following precautions are to be taken.

a) Water used in making up the solvent is boiled immediately before use to remove dissolved gases.

b) The solvent reservoir is held at a higher temperature (35 °C) than the column (25 °C) to keep the gas content of the eluting solvent low.

c) In order to prevent the column from running dry, a small magnetic valve is mounted below the stopcock *b* (see Fig. 1). Two copper electrodes are mounted about 2 cm above the cellulose surface in the solvent. These are connected to a relay which in turn can control the valve.
The valve is also connected with a time switch by which the fractionation can be stopped at a predetermined time.

Apparatus

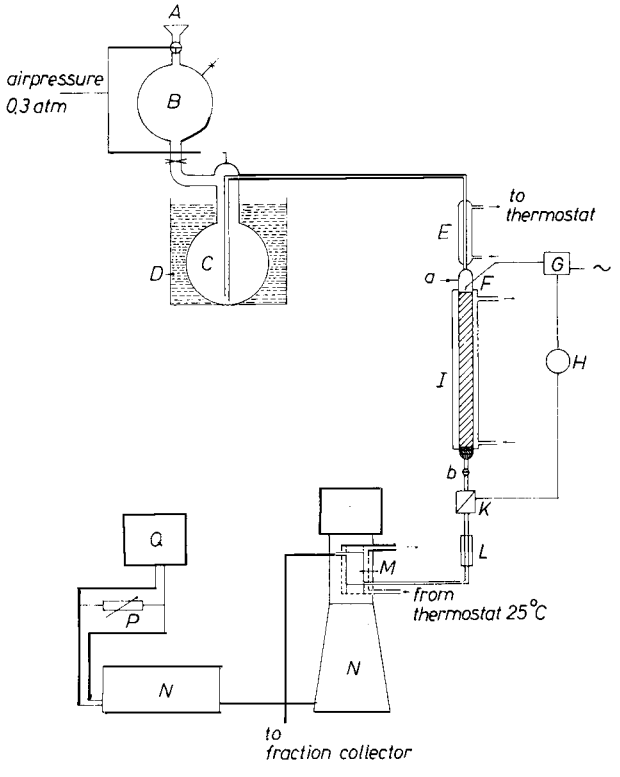

Fig.1

A filling funnel — *B* filling reservoir — *C* solvent reservoir — *D* constant temperature water bath 35 °C — *E* temperature conditioner — *F* copper electrodes — *G* relay — *H* time switch — *I* column — *K* magnetic valve — *L* filter — *M* thermostated flowthrough cell — *N* automatic polarimeter — *P* resistance box — Q recorder.

Analysis of Eluant

The optical rotation of the eluant is continuously measured by a Bendix-NPL type 143C automatic polarimeter[3]). The output of the polarimeter is recorded on a LKB-DC recorder[4]). The output terminals are shunted with a resistance of 800 Ω: a rotation of 0.0002° is visible on the recorder.

The light source of the polarimeter is filtered through a mercury green filter.

The eluant running out of the column is first filtered to remove possible glasswool cuts and air bubbles. It then flows through a 2 cm flowthrough cell. In order to prevent small deviations of the baseline of the recorded optical rotation the cell is held at a constant temperature by putting it in a close-fitting, thin, double-walled cylinder provided with two pipes which are connected to a thermostat of 25 °C.

When the eluant has passed the polarimeter cell, 25 ml fractions are siphoned by a LKB fraction collector. The fractions are marked by an event marker built in the LKB recorder. Thus the optical rotation of each individual fraction can be correlated with the recorded rotation.

Small amounts of CD which cannot be traced with the polarimeter are detected in the following way. Fractions which are supposed to contain traces of α or β CD are vaporised to dryness. Subsequently 1 gramme of water is added so that any residue that might be present is dissolved. Now 50 µl of this solution is applied to Whatman I paper, in the meantime restricting the applied solution to a small spot by continuous drying with a hairdryer. Finally, the paper is sprayed with a 1 % alcoholic iodine solution. α and β CD can now be detected by the appearance of a violet or yellow spot respectively.

Operation Conditions

In accordance with THOMA et al. (14), who investigated a mixture of homologous saccharides, we use 1-butanol, ethanol and water as the eluting solvent components. Since it is our intention to detect amounts of α CD in β CD and vice versa, we are able to use a more simple procedure. Clear separation is effected at 25 °C with a solvent of constant composition, viz. 1-butanol: ethanol: water = 42 : 29 : 29 (% by volume). Because large volumes of solvent are needed in fractionation the effect of using less pure 1-butanol and 96 % ethanol was investigated (see : materials). It appeared that the separation was of the same quality as when using analytical grade chemicals.

The flowrate of the eluting solvent is 75–90 ml/h and is almost constant during one run using a constant air pressure of approximately 0.3 atm. on the solvent reservoir.

The cellulose bed has a height of about 62 cm.

Operation of the Column

3 ml of a 4 % (w/w) CD solution in water, mixed with 3 ml eluting solvent is evenly and gently poured on to the cellulose so as not to disturb the surface. Pressure is now applied, allowing the liquid level to reach just

[3]) Bendix Electronics Ltd., New Basford, Nottingham (England).

[4]) LKB-Produkter AB, Stockholm (Sweden).

the cellulose surface. Now the pressure is released, about 3 ml solvent is poured on to the column and the pressure is reapplied until the solvent level just reaches the cellulose surface. Then this procedure is repeated. Finally, solvent is allowed to enter the column by opening stopcock *a* (Fig. 1) for a moment until the copper electrodes (F) are immersed in the solvent. A continuous feed of solvent is flowing out of reservoir C. When this reservoir is empty it can be refilled without interrupting the fractionation (see: Fig. 1). 1.7 l solvent is needed for one fractionation run lasting about 20 hours.

Results and Discussion

In Figure 2 a typical elution diagram is shown for a mixture consisting of 90 % α CD and 10 % β CD. The mixture is completely separated. This is controlled by vaporising the fractions between the two peaks (viz. fraction no. 47). No CD is found when applying the procedure described above (see: analysis of eluant).

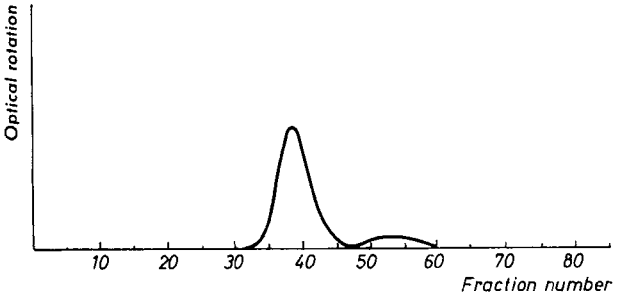

Fig. 2. Separation of α and β *CD*. Solvent 1-butanol : ethanol : water = 42 : 29 : 29 (% by volume). Sample 3 ml 3.6 % α *CD* + 0.4 % β *CD* (w/w). Flowspeed 80 ml/h. Fractions of 25 ml.

When a mixture consisting of 2.5 % β CD and 97.5 % α CD is fractionated, only a minor β CD peak can be observed. Smaller amounts of β CD were not detectable by means of the polarimeter. Very small amounts down to 0.5 % β CD in our α CD can be traced by means of the „paper"-method described above.

In the same way small amounts of α CD in β CD can be traced. A solution containing 3.98 % (w/w) β CD and 0.02 % (w/w) α CD has to be applied to the column. This is done by preparing a supersaturated solution. The elution curve is not a symmetrical one in this case, as can be seen in Figure 3.

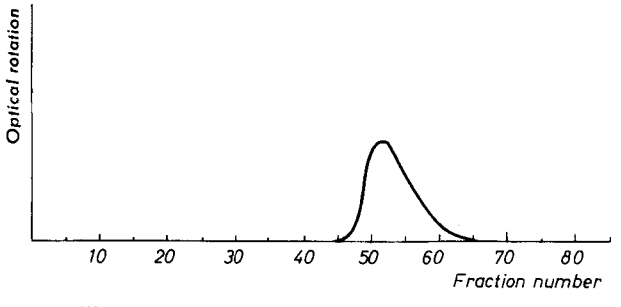

Fig. 3. Elution curve of β *CD*. Solvent 1-butanol : ethanol : water = 42 : 29 : 29 (% by volume). Sample 3 ml 3.98 % β *CD* + 0.02 % α *CD* (w/w). Flowspeed 79 ml/h. Fractions of 25 ml.

Since the iodine detection is more sensitive to α CD (violet) than to β CD (yellow) even less α CD can be traced with the „paper"-method. Amounts down to 0.3 % α CD in our β CD are detectable.

The area of the peaks and the weight of the residue after vaporising to dryness the fractions concerned, are a measure for the quantity of α and β CD respectively. We have not yet been able to obtain satisfactorily reproducible values of the peak areas of α and β CD, nor of the weights of residue, presumably because the water content of the original CD samples was not constant and unknown. Gas-chromatographic measurement of the water content by dissolving CD in anhydric dimethylformamide, which method will be described elsewhere, may perhaps solve this problem.

Conclusion

0.5 % β CD in α CD and 0.3 % α CD in β CD can now be traced by means of columnchromatography on cellulose. Since the concentration of CD in the eluant is determined polarimetrically, impurities other than optically active compounds cannot be observed. However, analysis of the refractive index of aqueous solutions (9) of the CD samples investigated may give information on this point.

Acknowledgements

The technical assistance of Mr. J. Hurkmans during the course of this investigation is gratefully acknowledged. The author feels greatly indebted to Prof. Dr. C. L. van Panthaleon van Eck and to ir. N. Wiedenhof for their critisism and interest shown throughout this study.

Summary

In order to find a good purity criterion for α and β cyclodextrin (Schardinger dextrin, cycloamylose, CD) with respect to each other a method using partition chromatography on cellulose columns has been investigated. This method allows of detecting 0.5 % β CD in α CD and 0.3 % α CD in β CD. A 0.12 g CD-mixture is needed for one fractionation run. One run is made within about 20 hours.

The detection of quantities down to 2.5 % α CD in β CD and vice versa can be made with an automatic polarimeter. Smaller amounts can be traced by concentrating the eluant, applying the solution on paper and spraying the paper with an alcoholic iodine solution.

Ein neues Verfahren zur Bestimmung der Reinheit von α- und β-Cyclodextrin durch Verteilungschromatographie an Cellulosesäulen

Zusammenfassung

Zur einwandfreien Bestimmung der Reinheit von α- und β-Cyclodextrin (Schardinger-Dextrin, Cycloamylose, CD), jeweils des einen vom anderen, wurde eine Methode geprüft, die auf der Verteilungschromatographie an Cellulosesäulen beruht. Mit dieser Methode lassen sich 0,5 % β-CD in α-CD und 0,3 % α-CD in β-CD nachweisen. Für einen Durchlauf benötigt man 0,12 g CD-Mischung und einen Zeitraum von etwa 20 Stunden.

Mengen bis hinunter zu 2,5 % α-CD in β-CD und umgekehrt können mit einem automatischen Polarimeter nachgewiesen werden. Kleinere Mengen kann man nach-

weisen, wenn man das Eluierungsmittel stärker konzentriert, die Lösung auf Papier trägt und das Papier mit einer alkoholischen Jodlösung besprüht.

Un nouveau Procédé pour déterminer la Pureté d' α- et de β-Cyclodextrine à l' Aide d' une Chromatographie à partition sur des Colonnes de Cellulose

Résumé

Pour déterminer d'une façon satisfaisante la pureté d'α- et de β-cyclodextrine (dextrine Schardinger, cycloamylose, CD), l'une relative à l'autre, on a examiné une méthode se servant d'une chromatographie à partition sur des colonnes de cellulose. Cette méthode permet de déceler 0,5 % de β-CD en de l'α-CD et 0,3 % d'α-CD en de la β-CD. Pour un passage de fractionnement, on a besoin de 0,12 g de mélange de CD. Un tel passage s'effectue en 20 heures environ.

Le décèlement de quantités plus grosses, descendant jusqu'à 2,5 % d'α-CD et de la β-CD et inversement, peut être accompli à l'aide d'un polarimètre automatique. Pour indiquer des quantités plus faibles, il faut concentrer l'éluant, appliquer la solution sur du papier et arroser le papier d'une solution alcoolique d'iode.

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(Arrived: January 14, 1967)

Effect of Phosphate Buffer on Dispersed Starch

I. Hydrolysis of Amylopectin at Various *p*H Values

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Introduction

Leloir, De Feckete, and Cardini (1) obtained evidence that uridine diphosphate glucose synthetase enzymes are located in or on the surface of the starch granule. Pottinger and Oliver (2) observed that if mild conditions are used in the isolation of the starch granule, then more enzymatic activity is retained. Under ideal conditions, the enzymes complexed with the starch granule may be quite active. Frequently starch granules are used to absorb alpha-amylase (3, 4). These examples illustrate that enzymes can be and are associated with starch granules and that some previously unexplainable experimental results (5, 6) may be due to enzymes adhering to the starch granule.

We shall show that an enzyme present in waxy starch granules hydrolyses the dispersed amylopectin at neutral *p*H when phosphate is added. The retention of the enzyme activity during dispersion is most likely due to the properties of the dispersing solvent (lithium plus guanidinium salts) and the mild conditions (room temperature) under which the granules are dispersed. Harrington and Schellman (7) have shown that concentrated solutions of lithium bromide, such as used in this study, protect proteins from being denatured. The hydrolysis of amylopectin observed under various conditions during the enzymatic study will be described.

Experimental

Preparation of Starch Samples.

Bear[1]) hybrid waxy corn starch was dispersed directly from the kernels, without isolating the starch granules. The kernels were first ground in a burr mill and then in water with a Virtis 23 homogenizer for approximately 5 minutes. About 50 ml of water were used for 10 kernels. The mash was centrifuged to separate the soluble protein from the starch granules and insoluble protein. The solids were then dispersed at room temperature by using a solvent consisting of 5 M lithium thiocyanate plus 1.5 M guanidinium thiocyanate or 6 M lithium bromide plus 2 M guanidinium chloride. Both solvents give the same results (8). Although the dispersion of the 2 % starch was complete within 4 hours, the dispersion was continued for approximately

[1]) Bear Hybrid Corn Co., Inc., Decatur, Illinois, U.S.A. The mention of firm names or trade products does not imply that they are endorsed or recommended over other firms or similar products not mentioned.