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Note

Chromatographic determination of cyclodextrins on benzoylated polyacrylamide gels

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Cyclodextrins (CDs; specifically α-, β- and γ-CDs) are cyclic dextrins produced enzymatically from starch on an industrial scale. Concomitantly with the increase in the applications of CDs in various fields of science and technology, their analysis is becoming more and more important[1].

CDs have been analysed by complexation with specific colours[2,3], by enzymatic methods[2,3] and by high-performance liquid chromatography (HPLC) with aqueous acetonitrile as the mobile phase[4,5]. The first two types of method may suffer from certain interferences and are most suitable for routine applications where the sample composition maintains relatively constant[3]. Whenever HPLC is applied to the study of enzymatic conversion processes of CDs from starch, the sample preparation is tedious because the other dextrinous side-products must be removed. The hydrolysis of these side-products by glucoamylase[6] to glucose is laborious and contains a risk of partial disappearance of especially γ-CD[3].

We describe here a specific method for the quantitation of individual CDs based on an affinity chromatographic separation mechanism. Bio-Gel P-6 was aminated and the resulting gel benzoylated. This gel effected the separation of CDs from partially hydrolysed starch and linear maltooligosaccharides and can be utilized for the analysis of CD preparations without any special sample preparation.

EXPERIMENTAL

Preparation of benzoylated Bio-Gel

A 25-g amount of Bio-Gel P-6 (Bio-Rad Labs.) was added to 500 ml of ethylenediamine (technical grade, E. Merck) in a round-bottomed flask equipped with a reflux condenser and a magnetic stirrer. The suspension was kept at 110–115°C (oil-batch) with continuous stirring for 4 h. The mixture was cooled, filtered and washed thoroughly with water, 0.2 *M* sodium chloride–0.001 *M* hydrochloric acid, water, 10% ethanol and water. The gel contained 0.57 mmol of amino groups per gram of suction-dried moist gel (potentiometric titration). The method is a modification of that presented earlier[7,8].

Benzoic acid (1 g) was dissolved in 30 ml of warm water and the pH was adjusted to 4.7 with 1 *M* sodium hydroxide solution. If all the benzoic acid does not dissolve, a minimum amount of methanol may be added. Suction-dried aminated

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Bio-Gel P-6 (30 g) was added and the pH was readjusted to 4.7 (with hydrochloric acid or sodium hydroxide). N-Ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrochloride (Sigma) (1.5 g) was dissolved in 5 ml of water at 0°C and the solution added dropwise to the gel suspension at room temperature during 30 min with continuous stirring. The pH was maintained at 4.7 with hydrochloric acid. Gentle stirring was continued for 4 h and the gel filtered off and washed with water, 50% ethanol, methanol, 50% ethanol, water, 0.2 *M* sodium chloride solution and water (1 l each). According to potentiometric titration, 20–25% of the amino groups were benzoylated.

CD samples

Cyclodextrins were purchased from Sigma and were dissolved in distilled water (5 mg/ml each). The conversion reaction of starch to CDs was carried out by incubating purified cyclomaltodextrin glucanotransferase[9] with pregelatinized potato starch (E. Merck) in 20 m*M* imidazole–hydrochloric acid buffer (pH 6.8) containing 1 m*M* calcium chloride. The starch concentration was 10% (w/v) and the amount of enzyme was 100 units[9] per gram of starch. The reaction took place at 60°C and it was stopped after 2–12 h with 10-min boiling. These mixtures were subjected to analyses for CDs, unless stated otherwise.

Analysis

The equipment involved a peristaltic pump (Pharmacia P-3), a benzoylated gel column (160 × 14 mm I.D.) and a refractive index (RI) detector (LKB Model 2152). Samples of 0.1–0.5 ml were applied and the elution was carried out with distilled water (flow-rate 30 ml/h).

HPLC analyses were performed with an LKB HPLC system including a refractive index (RI) detector as above and a carbohydrate analysis column (Waters-Millipore; No. 84038). Acetonitrile–water (65:35, v/v) was used as the mobile phase at a flow-rate of 1.0 ml/min. The samples (0.5 ml) of the conversion mixtures were prepared by chromatography on Trisacryl GF-05 (LKB) column (40 × 2.1 mm I.D.) combining all fractions containing CDs (100% recovery as measured by specific colour reactions[3]). The elution was carried out with water (flow-rate 30 ml/h).

RESULTS

Studies of the preparation of samples for HPLC

Normally few problems are encountered when pure CDs or mixtures of CDs with maltooligosugars of G1–G8 (glucose = G1, maltose = G2, etc.) are analyzed by HPLC[4,5]. When conversion mixtures of starch are dealt with, at least the bulk of the dextrins should be removed as they are not eluted and hence rapidly degrade the HPLC column. In spite of this fact, HPLC methods with unpurified samples have been reported, however[10].

We studied the sample preparation of conversion mixtures using various techniques. Higher maltosugars were removed by ultrafiltration with an Amicon cell (PM 5 membrane) while the filtrate was analysed by HPLC. Fig. 1 shows that the concentration of CDs in the filtrate is largely dependent on the volume filtered and almost all of the solution must percolate through before reliable results are obtained.

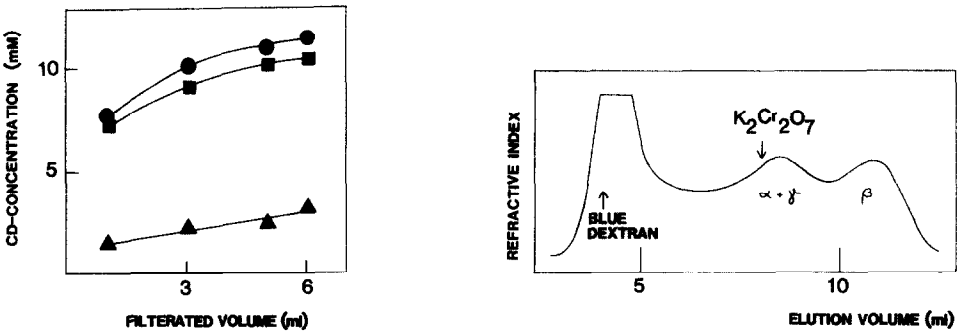

Fig. 1. HPLC analysis of CDs in the permeate through an Amicon PM 5 ultrafiltration membrane as a function of percolating volume. The enzymatic conversion mixture of starch (see Experimental) was diluted 10-fold with water prior to the filtration at room temperature at a rate of about 1 ml/min. ● = β-CD; ■ = α-CD; ▲ = γ-CD.

Fig. 2. Separation of enzymatic conversion mixture of starch, containing all three CD forms, on Trisacryl GF-05 gel (for details, see Experimental). The CDs were identified on the chromatogram by separate runs with pure CDs and by measuring the CDs with specific colour reactions[3]. The elution of Blue Dextran 2000 and potassium dichromate are indicated to show the positions of high- and low-molecular-weight dextrins, respectively. As β-CD is retained from non-excluded sugars it is specifically adsorbed on the gel.

Various gel chromatographic separations were also tested. On Sephadex gels G-10–G-50 (Pharmacia) adsorption of dextrins occurred resulting a poor resolution. Whereas Trisacryl GF-05 as a rule showed a low degree of adsorption of dextrins, β-CD was specifically retarded on this material (Fig. 2). This behaviour is probably due to the formation of an inclusion complex between β-CD- and trishydroxymethylmethane functions which could be of appropriate molecular size to be imbibed. As a result, a long elution time is required. Attempts at the specific precipitation of acyclic dextrins with organic solvents were not satisfactory. With CDs, one should be careful when using commercial disposable HPLC sample preparation kits as the CDs may become specifically attached to their packing materials and hence one or more of the CD forms may be lost.

Affinity separation of CDs

Our study was primarily conducted in order to achieve a convenient method for the determination of CDs in enzymic conversion mixtures of starch. In the early stages commercially available phenyl-glass (μBondapack phenyl; Waters Assoc., product number 27198) was observed to separate CDs with a specific retention from other sugars. Broad diffuse peaks appeared for CDs, and although elution with water was used, sample purification was needed in order to keep the column lifetime reasonable. Therefore, we decided to seek an affinity matrix combining controlled size-exclusion and affinity separation mechanisms. This system should be beneficial as then the capacity for CDs is at its maximum while little binding of large dextrins occurs owing to a limited adsorptive surface.

The problem in designing a gel-type affinity matrix having a high capacity concomitantly with mechanical strength is evident. Polyacrylamide gels were studied in more detail to find the proper degree of substitution and porosity. The degree of

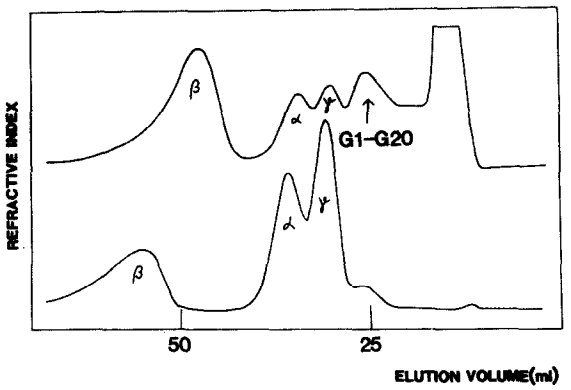

Fig. 3. Chromatography of a standard mixture of pure CDs (bottom curve) and of an enzymatic production mixture of CDs (top curve) on benzoylated Bio-Gel P-6. Sample sizes, 0.25 ml for the pure CD mixture (0.5 mg/ml of each CD) and 0.5 ml for the conversion sample. The region for the elution of oligosugars containing 1–20 glucose units is indicated by G1–G20.

benzoylation was critical; the best results were obtained with gels with 10–25% benzoylation of the amino groups. When the substitution was higher, β-CD could not be eluted from the column with water. The porosity of the gel was another adjustable parameter. A substitution-dependent shrinkage of the gel with concomitant changes in the porosity took place during the syntheses. We found experimentally that the synthesis and starting materials described here produced a satisfactory compromise to be used with water elution at room temperature. However, the support and the method provide several possibilities for further improvement.

Fig. 3 shows the elution patterns of CD standards and of a conversion product from starch on benzoylated polyacrylamide gel. Characteristic of the chromatographic system is the diffuse rear sides of the β-CD peaks and the dependence of the elution volumes on the CD concentration. As suggested above, greater benzoylation increased the elution volumes of CDs. Buffer ions, not competing with CDs for the complexation, increased the elution volumes, as did lowering of the temperature of the chromatography. The tailing of the peak of β-CD appeared in all tested conditions. The flow-rate did not affect the peak shapes and thus the adsorption isotherms of CDs should be convex. In general, such systems are unfavourable for resolution but in the context of the separation of only three molecular species from each other, the situation is not so harmful.

The elution pattern of the standard CDs (Fig. 3) shows a complete separation of β-CD and a partial separation of α- and γ-CDs. It also shows two minute impurity peaks. Direct analysis of the same standards by the HPLC method[4,5] did not reveal their presence. Hence the affinity method provides a positive means of analysing small amounts of impurities among analytical-grade CDs including inorganic salts. This finding should be of considerable importance in analyses of CDs produced for, *e.g.*, medical purposes.

High- and low-molecular-weight dextrins are separated on the gel in Fig. 3 by a size-exclusion mechanism. This is additional information obtained from the starch conversion mixtures and can be utilized in determining the degree of hydrolysis of starch in industrial processes.

TABLE I

COMPARISON OF RESULTS OBTAINED BY AFFINITY AND HPLC METHODS

The conversion mixtures for the production of CDs were prepared as described under Experimental; the reaction times were 2 h (sample 1) and 12 h (sample 2). Prior to HPLC on a Waters Assoc. carbohydrate analysis column, the samples were purified on Trisacryl GF-05. Using HPLC, the recovery of pure CDs was 97–100% (glucose as the internal standard). Results are in mg/ml measured at peak height.

Method	*Sample 1*			*Sample 2*		
	α-CD	*β-CD*	*γ-CD*	*α-CD*	*β-CD*	*γ-CD*
Benzoylated Bio-Gel P-6	0.17	2.90	0.64	0.58	2.15	0.44
HPLC	0.15	2.99	0.67	0.65	2.23	0.51

Table I compares the analyses of two conversion mixtures by the affinity and HPLC methods (for details, see Experimental). The differences between the results are small enough to fall inside presumable experimental errors, even though there is some tendency for higher values to be obtained with the HPLC method.

DISCUSSION

The determination of CDs is important in the quality control of various products containing them and in the enzymatic production of CDs from starch. For these diverse areas the most appropriate analytical methods should be found. A complicating factor is the sample preparation, as many materials adsorb CDs considerably differently depending on their individual forms.

The advantages of the affinity method described here over the existing HPLC method are as follows: (1) sample preparation is unnecessary; (2) positive quantitation of minor impurities (large dextrins, salts) in the CDs is achieved; (3) the whole procedure can be automated more easily; (4) the method does not employ harmful solvents; (5) elution with water increases the sensitivity of refractive index detection; (6) acyclic dextrins of any size do not overlap with CDs; (7) an estimate of the amount of dextrins is obtained in the hydrolysis stage of starch. In contrast, the HPLC method has the following advantages: (1) maltosugars of G1–G10 can be analysed with CDs; (2) the analysis step is faster.

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REFERENCES

1 J. Szejtli, *Chimica Oggi (Maggio)*, (1987) 49.
2 M. Mäkelä, T. Korpela and S. Laakso, *J. Biochem. Biophys. Methods*, 14 (1987) 85.
3 M. Mäkelä, T. Korpela, J. Puisto and S. Laakso, *J. Agric. Food Chem.*, 36 (1988) 83–88.
4 B. Zsadon, K. H. Otta, F. Tüdös and J. Szejtli, *J. Chromatogr.*, 172 (1979) 490.
5 H. Hokse, *J. Chromatogr.*, 189 (1980) 98.

6 T. Kato and K. Horikoshi, *Biotechnol. Bioeng.*, 26 (1984) 595.
7 J. K. Inman and H. M. Dintzis, *Biochemistry*, 8 (1969) 4074.
8 K. K. Inman, *Methods Enzymol.*, 34 B (1974) 30.
9 M. J. Mäkelä and T. K. Korpela, *J. Biochem. Biophys. Methods*, 15 (1988) 307–318.
10 M. Sato, Y. Yagi, H. Nagano and T. Ishikura, *Agric. Biol. Chem.*, 49 (1985) 1189.