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Note

High-performance liquid chromatographic analysis of the products of enzymatic lactose hydrolysis

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A sufficiently complete analysis of the products of enzymatic lactose hydrolysis in whey or other dairy fluids is of primary importance not only for the investigation of this reaction in the laboratory but also for effective control of an industrial process. It is known that besides the main hydrolysis products, glucose and galactose, some unwanted side products, namely oligosaccharides (OS), are formed. This is due to the transgalactolytic activity of the same enzyme, β-galactosidase, which is used for the hydrolysis.

At present the analysis of the products is mostly limited to the enzymatic determination of glucose alone, or in some cases glucose and lactose. Other components of the reaction mixture are usually ignored either because of the tedious and time-consuming methods needed, or because of insufficient knowledge of the reaction mechanism.

Comparison with other methods

In a number of recent studies[1–3] paper chromatography was used for the determination of OS formed in enzymatic lactose hydrolysis. Separation of the sugars posed no problems, however the quantitative evaluation was difficult. The same authors together with others[4,5] therefore additionally used gas–liquid chromatography (GLC). However, the interpretation of the results was very difficult due to multiple response peaks for one substance and its enantiomers. Also the samples must be chemically modified, silanized, adding a further degree of complexity. An excellent application of GLC in complex sugar analysis was presented by Nikolov and Reilly[6] in their study of enzymatic cellulose hydrolysis. However, both paper and gas–liquid chromatography require at least ten times more time than the presented HPLC method.

The analysis of glucose alone can be done in less than 1 min by an automatic glucose analyser. Lactose can also be determined enzymatically, *e.g.*, Test No. 176 303, Boehringer, Mannheim, F.R.G.) in combination with galactose determination. After the first galactose determination the sample is completely hydrolyzed by β-galactosidase and the galactose test is performed again. The amount of lactose can be calculated from the difference in results between the two galactose tests. In this way the three main sugar components can be determined, but three different time-consuming procedures have to be used. More important, all the OS will be destroyed

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by the total hydrolysis and added to the apparent lactose content. Depending on the enzyme source, the formation of OS can be very prominent so that a stoichiometric determination of one sugar from another may be very misleading[7]. Owing to the rapid development of HPLC techniques, it has recently become possible to analyse complex sugar mixtures with a reasonable time and effort.

The specific objective of this work was to demonstrate the present capacity of HPLC for the analysis of complex sugar mixtures using as an example the determination of oligosaccharides (OS) which are formed besides glucose and galactose in the process of enzymatic lactose hydrolysis. Until now, the HPLC analysis of sugars has been done using mostly an amino-bonded phase, *e.g.*, LiChrosorb-NH_2 (Merck, Darmstadt) and acetonitril–water as the mobile phase. By varying the solvent ratio, a very good separation of monosaccharides from each other as well as di-, tri-, and higher sugars can be achieved. On the other hand, it is quite difficult to obtain a good separation of monosaccharides and higher sugars at the same time. The separation of glucose and galactose is usually incomplete. A better separation can be obtained using the new column Sugar PAK I (Waters Associates). The peaks of monosaccharides as well as of other higher sugars are clearly separated so that glucose, galactose, lactose and OSs can be determined in one run taking *ca.* 10–12 min. In this manner a number of analyses can be made in a reasonable time. Thus, the quantitation of the most important OSs can be made, allowing fundamental information about the mechanism and kinetics of enzymatic lactose hydrolysis to be obtained.

The Waters Sugar PAK I column is a microparticulate gel ion-exchange bed designed for chromatography of sugars especially in beet, cane and corn processing plants to separate glucose from higher oligomers found in typical corn syrups. From the following it is seen that its use can be successfully extended into the area of enzymatic lactose hydrolysis.

EXPERIMENTAL

The isocratic HPLC apparatus consisted of the following: mobile phase reservoir; pump M-45 (Waters Associates, Milford, MA, U.S.A.); automatic sample injection, Waters Intelligent Sample Processor (WISP) 710B, precolumn, Waters Associates guard column filled with Aminex Q15S Ca^{2+}, or Bio-Rad Micro Guard, disposable cartridge, Carbohydrate (Bio-Rad Labs., Richmond, CA, U.S.A.); chromatographic column, Sugar PAK I (Waters Associates), 300 × 6.5 mm I.D.; refractive index detector, Interference refractometer Multiref 902 (Optilab, Vallingby, Sweden); thermostats for separate thermostating of the column and refractometer, Haake F3M,S (Haake Mess-Technik, Karlsruhe, R.R.G.); recorder, PM 8010 (Philips, Eindhoven, The Netherlands); integrator, HP 3390A (Hewlett-Packard, Avondale, PA, U.S.A.).

The mobile phase comprised twice distilled water, filtered through a 0.45-μm Micrometer Metricel membrane (Gelman) and degassed. Calcium acetate was sometimes added to regenerate the column.

The flow-rate was typically 0.5 ml/min and the pressure drop less than 10 MPa. The column was in an aluminium block thermostated at 90°C; the detector was kept at 75°C. The samples were diluted to a 1–2% sugar concentration and filtered through

a 0.2-μm Micrometer Metricel membrane filter to remove particles and to avoid microbial contamination. The injection volume was typically 10–20 μl.

RESULTS

A chromatogram of a solution used as an external standard consisting of glucose, galactose, lactose for diaccharides and raffinose for trisaccharides (Fig. 1) shows a good separation of all the peaks. It was found that an integral of the response area was a linear function of the sugar concentration. The time required for one analysis was less than 11 min.

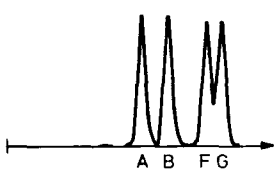

Fig. 1. Chromatogram of the calibration solution used as an external standard. Column: Sugar PAK I. Sample: 10 μl standard solution of raffinose (1%), lactose (1%), glucose (1%) and galactose (1%). Mobile phase: water, 0.5 ml/min, 90°C. Detector: RI Optilab 902, 75°C. Integrator: HP 3390A. Results: A, raffinose, retention time 6.17 min, amount 10.077 g/l; B, lactose, 7.38 min, 10.081 g/l; F, glucose, 9.18 min, 10.024 g/l; G, galactose, 9.85 min, 10.189 g/l.

The three chromatograms in Fig. 2 are representative determinations during enzymatic lactose hydrolysis under various conditions. The first peak belongs to the buffer salts (samples were not deionized), followed by the peaks of oligosaccharides in descending order. The last two peaks to the right are due to glucose and galactose. The samples were taken at the degree of hydrolysis at which the maximum amount of OS was observed.

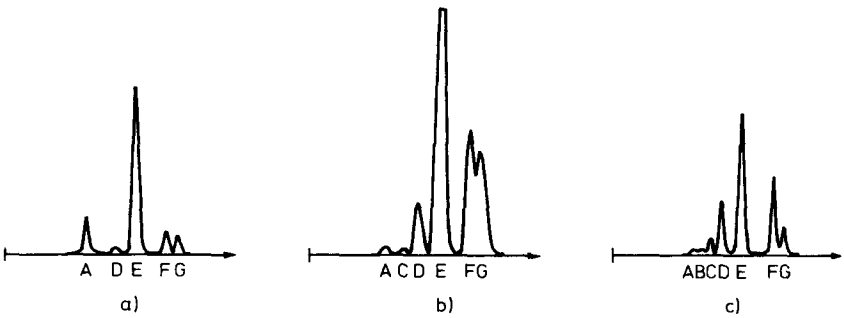

Fig. 2. Representative examples of the Sugar PAK I chromatograms of lactose hydrolysates. The degree of hydrolysis, X, corresponds to maximum OS production. Peaks: A = salts; B = penta-, C = tetra-, D = tri-, E = disaccharides; F = glucose; G = galactose. a, Catalyst: β-galactosidase from *Aspergilus niger* immobilized on Duolite, activity 100 AU/g, 5 g in 25 ml of lactose solution; initial lactose concentration, 4.8%; X = 0.34. b, Catalyst as in a; initial lactose concentration, 15%; X = 0.52. c, Catalyst: β-galactosidase from *A. oryzae*, 80 mg per 100 ml lactose; initial lactose concentration, 30%; X = 0.59.

DISCUSSION

The main purpose of lactose hydrolysis is usually to increase its value by conversion into a glucose/galactose syrup which has, in addition to much higher sweetness, a number of advantages[8]. Hence, any side products like OS are undesirable and their content in the final product must be minimized by a suitable operating policy. The conditions of such a policy, the process control and the characterization of the end product require precise analysis of all important reaction components within a reasonable time. Until now, little more than one "key" compound, usually glucose, was determined, mostly due to the lack of methods suitable for routine measurements.

With the advent of the new carbohydrate columns HPLC has become suitable for the routine determination of all the important sugars. The Sugar PAK I column appears to be ideal for the separation and determination of the main lactose hydrolysis products glucose, galactose, the disaccharides and the OS up to pentasaccharides. Fig. 3 shows an example of enzymatic lactose hydrolysis as a function of time. The high OS content was caused by the high initial lactose concentration and also the enzyme type from *Aspergilus oryzae* used, which was found to produce un-

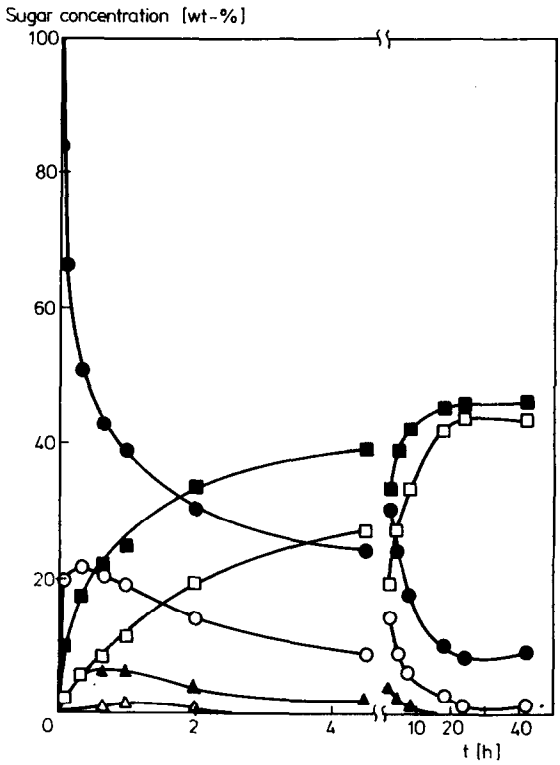

Fig. 3. Batch lactose hydrolysis. Catalyst: β-galactosidase from *A. oryzae*. Initial lactose concentration: 30%. Temperature: 50°C. McIlvaine buffer, pH = 4.5. Curves: ■, glucose; □, galactose; ●, disaccharides; ○, trisaccharides; ▲, tetrasaccharides; △, pentasaccharides.

usually high amounts of OS. The main advantages of this column are the short retention time and the possibility of simultaneous separation of monosaccharides and oligosaccharides. A simple sample preparation prior to injection is another positive aspect.

At present, therefore, HPLC with the Sugar PAK I column seems to be the best method for the monitoring of enzymatic lactose hydrolysis in the laboratory as well as in industrial practice. For the kinetic modelling of the process it would be interesting to have a column which would separate also disaccharides, mainly because an intermediate allolactose seems to play an important rôle in the reaction mechanism[4,9]. Despite the poor monosaccharide separation and long retention times, the NH_2 column which was used earlier[10,11] can be useful for the separation of di- and trisaccharides. Perhaps an acetonitrile–water gradient would yield a better separation of monosaccharides and OSs.

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