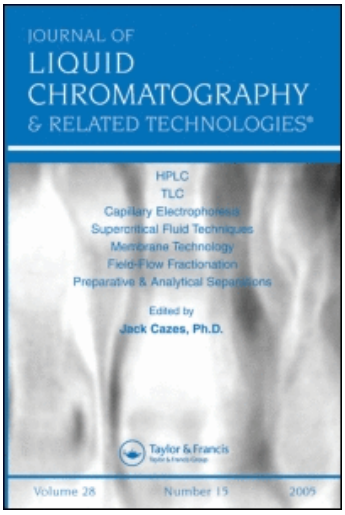

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Measurement of Cellulolytic Activity by Low Pressure Liquid Chromatography

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To cite this Article Ladisch, Michael R. , Anderson, Allen W. and Tsao, George T.(1979) 'Measurement of Cellulolytic Activity by Low Pressure Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 2: 5, 745 — 760
To link to this Article: DOI: 10.1080/01483917908060100
URL: http://dx.doi.org/10.1080/01483917908060100

JOURNAL OF LIQUID CHROMATOGRAPHY, 2(5), 745-760 (1979)

MEASUREMENT OF CELLULOLYTIC ACTIVITY BY LOW PRESSURE LIQUID CHROMATOGRAPHY

by

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Abstract

The application of aqueous, low pressure liquid chromatography to the assay of cellulolytic enzyme activity is discussed. The advantages of this method are speed of analysis (less than 20 min.), small sample size (20 μℓ), good resolution, and a tolerance of the system to the presence of extraneous salts and proteins. Examples showing the use of this tool are given.

INTRODUCTION

Basic research on the enzyme catalyzed hydrolysis of cellulose has resulted in the development of techniques for measuring cellulolytic activity. Methods representative of these techniques are summarized in Table

1. While each technique which is listed in the table has its advantages, liquid chromatography (LC) appears to be the most versatile approach, since identification, as well as quantification, of the individual products of hydrolysis are possible with this method. Furthermore, the advent of modern, commercially available LC hardware makes it possible to carry out analyses quickly and easily.

There are two approaches which are available for LC of carbohydrates:

(1) reverse-phase or partition chromatography,

and

(2) aqueous chromatography.

Reverse-phase silica packings capable of withstanding high pressure are used with an organic/aqueous eluent such as acetonitrile-water (10). Similarly, ion-exchange resins with ethanol-water as eluent give excellent results as demonstrated by Samuelson et al. (11,12). However, partition

Table 1.

Representative Methods for Detection of Cellulolytic Activity

Method	Description	Reference
Filter Paper	Incubation of cellulolytic enzymes with filter paper followed by reducing sugar assay.	1
Fungal Growth on Cellulose-Azure	Cellulolytic fungi release blue color from Cellulose-Azure	2
Incubation w/cellodextrins	Enzyme incubated with cello-dextrins. Products measured by separation on chromatography column and colorometric assay for sugars	3
Incubation w/cellodextrins	Total sugars formed measured by colorometric methods.	4,5,6,7
High Pressure, Reverse Phase, Liquid Chromatography	Cellodextrin hydrolysates separated using reverse phase LC with refractive index detector.	8
Low Pressure Aqueous Liquid Chromatography	Cellulose and cellodextrin hydrolysates separated using aqueous phase LC with refractive index detector.	9

chromatography has limitations. The high cellodextrins (cellotetraose through celloheptaose) have low solubility in solvents which are only partially aqueous (13). Furthermore, carbohydrate samples containing salts or proteins have to be cleaned up since the salt and proteins tend to precipitate in a semi-aqueous solvent.

The samples analyzed in our laboratory contained significant quantities of high-molecular-weight oligomers as well as (buffer) salts and (enzyme) protein. The salts and protein were difficult to remove without affecting the carbohydrate concentration in the sample. Initially both partition chromatography (using commerically available columns with acetonitrile-water as eluent) and chromatography with cation-exchange resin (with water as the sole eluent) were tried. The latter approach was found to be preferable for our work.

MATERIALS AND METHODS

Packing

The packing material used to make the LC columns discussed in this report is Aminex 50W-X4 (Bio-rad Laboratories, Richmond, California, USA), a 4% cross-linked styrene divinyl-benzene cation exchanger in the Ca^{++} form. This material was purchased in the H^{+} form (20 to 30 μm in diameter) and then sized and converted as described in a previous report (14).

The ion exchange resin was packed in a 6 mm i.d. X 60 cm column using a Haskel pneumatic amplifier pump. Details of the packing procedure are given elsewhere (9,14).

Column Operation

After packing, the column is connected to the liquid chromatograph and heated for 2 h at 85°C. The chromatograph was a Waters Assoc. ALC/GPC 201 series instrument with a U6K injector, an M6000A pump controlled by a Model 660 flow controller, and a differential refractometer detector

connected to a Spectra-Physics Autolab I programmable integrator and an Omniscribe strip chart recorder. The detector was thermostated to 30° with a Model FE Haake circulating water-bath.

The flow of degassed, distilled water through the column is initiated over a 20 min period, using Program No. 4 on the Model 660 flow controller. The water is kept degassed by maintaining it at 85 to 90° with continuous stirring in a 1-l solvent reservoir flask. The water passes through a solvent reservoir filter (20 to 30 μm) and then a 1-m coil of 3.2-mm I.D. PTFE tubing before reaching the pump. The tubing, which is suspended in air at room temperature, cools the water to room temperature. Once started, water is kept running through the column at a constant flow-rate 24 hours per day. All analyses were carried out at a constant flow-rate using water as the eluent.

At a flow on the order of 0.5 ml/min (1.8 cm/min), the pressure drop for this type of column is 100 to 250 p.s.i.g. and the plate height is 0.11 mm. Separation qualities typical for this type of column for cellodextrins is shown in Figure 1. The resolution is good and (sample to sample) injection time is reasonable (13.5 min).

Other attractive features of this type of column include a tolerance toward salts and proteins dissolved in the sample and a satisfactory operational stability. Some columns in our laboratory have withstood over 2600 hours of continuous use. One other advantage of this approach to LC is the low operational pressure of the system. This simplifies matters since high pressure-pumps, plumbing, and injection hardware, although sufficient, may be replaced by low pressure components, if desired.

APPLICATION TO ANALYSIS OF CELLULOLYTIC ACTIVITY

The use of liquid chromatography is appropriate for the analysis of cellulose solubilizing enzyme activity in fermentation broth, the measurement of activity of the cellulase complex, and the study of the kinetics

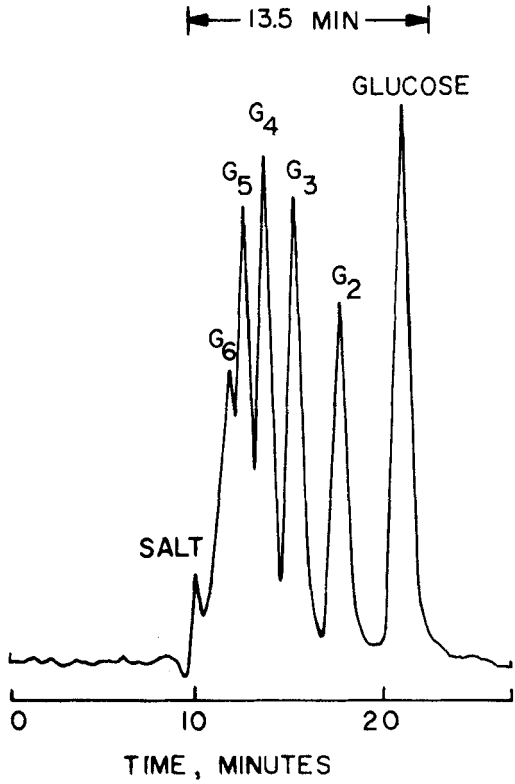

Fig. 1. Separation of cellodextrins on Aminex 50W-X4 using water as sole eluant. Flow = 0.5 ml/min (1.8 cm/min),

and mode of action of pure component enzymes. Since the columns described in the MATERIALS AND METHODS section can tolerate moderate concentrations of salts and protein, sample clean-up in most cases is not required. The utility of LC will be apparent in the examples given below.

Activity of Fermentation Broth

The production of a cellulytic enzyme by a micro-organism such as Trichoderma reesei may be readily monitored using a combination of the filter paper assay (1) and liquid chromatography. The measurement of enzyme as a function of time is important to the determination of optimum fermentation conditions.

A typical procedure for measuring enzyme activity in a fermentation broth is as follows. The broth is sampled and suspended solids are centrifuged down. The supernatant is then combined with buffer and filter paper as described in reference (1). After incubation, however, the supernatant is injected into the LC rather than being subjected to a reducing sugar assay. Chromatograms of appropriately diluted enzyme broth (i.e., the blank) and the filter paper hydrolysate are compared in Figure 2. The salts and protein elute first with sugars (cellobiose and glucose) following. No sample preparation was required other than spinning down suspended solids.

It should be noted that this method shows only the soluble sugars formed. The reducing ends on the cellulose which might be formed by enzyme activity are not accounted for. Thus, the apparent enzyme activity given by LC analysis of soluble sugars will be less than that given by a reducing sugar assay which includes the non-soluble cellulose portion as well as soluble sugars. The activity of the enzyme giving the chromatograms in Figure 2 was about 1 IU/ml (1 IU = 1 μmole sugars formed as glucose/min).

Activity of Cellulolytic Complexes

Knowledge of the product distribution as well as the quantity of sugars formed by various cellulolytic complexes is useful since not all sources of cellulases have the same ratio of glucanohydrolase/cellobiohydrolase/cellobiase activity. An interesting comparison is given by cellulases from two strains of T. reesei: QM9414 and NG14. NG14 is a mutant recently reported by researchers at Rutgers University (15).

The procedure followed for the two enzyme preparations was to combine 100 μℓ of enzyme (containing 100 mg enzyme/ml) with 1.4 ml of buffer (pH 4.8, Sodium acetate) and incubating with either:

(a) 50 mg filter paper (1),

(b) a filter disc weighing c 50 mg (15),

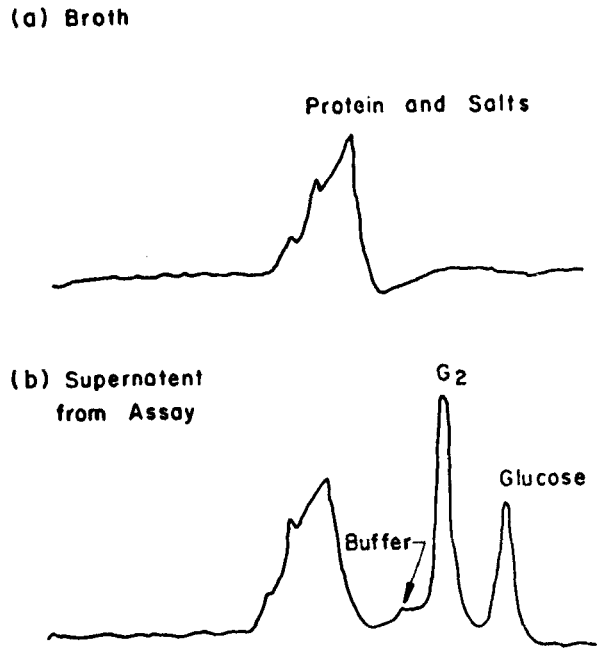

Fig. 2. Chromatogram of (a) fermentation broth, and (b) hydrolysate from action of broth on filter paper.

or

(c) 50 mg of microcrystalline cellulose (Avicel®).

After 1 hour incubation at 50°C in the case of (a) or (b), or 2 hours in the case of (c), the mixture was spun down and injected into the LC.

The chromatograms for QM 9414 and NG 14 are given in Figure 3 and Figure 4, respectively. Data obtained from these chromatograms is summarized in Table 2. The data show that the NG 14 preparation is more active than QM 9414 for filter paper and Avicel® but not for filter discs. It appears that, for both enzymes, the activity on filter discs is lower than for filter paper or Avicel®. In other words, the solubilizing activity measured for a particular enzyme preparation, appears to be a function of substrate.

Another interesting feature shown by these chromatograms is the absence of any significant amounts of higher oligosaccharides. The data also show

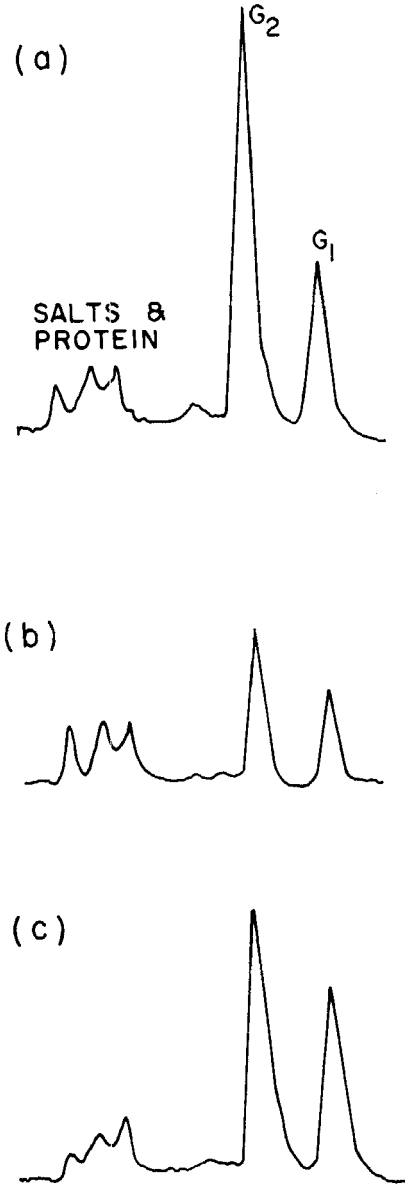

Fig. 3. Chromatograms of QM 9414 hydrolysates of (a) filter paper, (b) filter discs, and (c) Avicel[R].

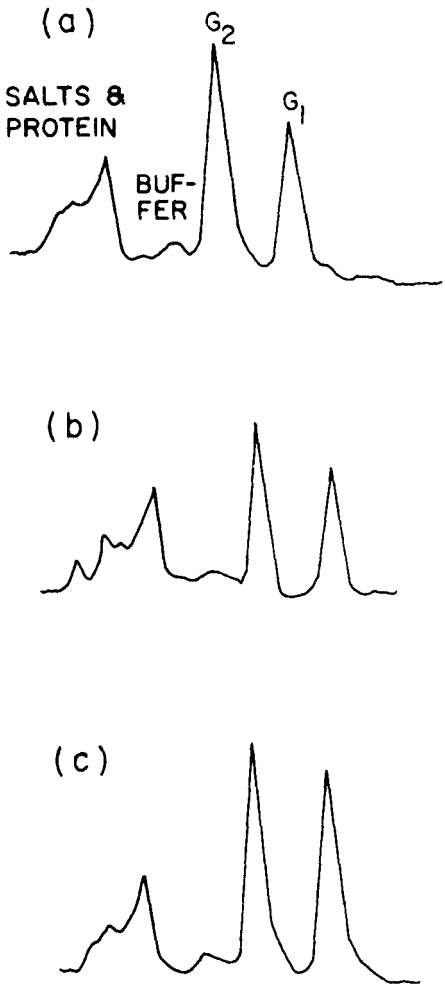

Fig. 4. Chromatograms of NG 14 hydrolysates of (a) filter paper, (b) filter discs, and (c) Avicel ^{R} .

that NG 14 does not have as high a cellobiose hydrolyzing activity as QM 9414 (see G_2/G_1 ratios in Table 2).

This example gives an indication of the usefulness in detecting cellulolytic activity by LC. Both quantitative and qualitative differences between enzyme preparations are easily discerned with this tool.

Table 2.

Comparison of Activity of QM 9414 vs. NG 14

	QM 9414	NG 14
Filter Paper		
Conversion (%)[+]	10.	15.
Activity (IU/mg)[++]	55×10^{-3}	85×10^{-3}
Ratio G_2/G_1[*]	0.74	1.2
Filter Disc		
Conversion (%)[+]	6.4	4.5
Activity (IU/mg)[++]	33×10^{-3}	24×10^{-3}
Ratio G_2/G_1[*]	0.57	0.82
Avicel[R]		
Conversion (%)[+]	11.	13.
Activity (IU/mg)[++]	29×10^{-3}	35×10^{-3}
Ratio G_2/G_1[*]	0.53	0.67

[+]Based on cellulose; corrected for weight increase due to water addition upon hydrolysis.

[++]1 IU = μmoles soluble sugars formed as glucose/min. Mg refers to mg enzyme powder.

[*]Mole G_2/Mole G_1 where G_2 = cellobiose and G_1 = glucose.

Study of Kinetics and Mode of Action

The study of kinetics and mode of action of cellulases requires:

(1) pure, homogeneous enzyme components,

(2) pure substrate,

and

(3) analytical techniques for detecting and quantifying the products of hydrolysis.

The first condition is a difficult one, and it is the subject of much research in the cellulase field. The second condition is easy to satisfy, if insoluble cellulose substrate is used. If soluble cellodextrins are required, this condition is more cumbersome. However, a method is available where gram quantities of cellodextrins in water may be prepared (16). One way of satisfying the third condition is low pressure liquid chromatography using water as eluent.

The method discussed in this paper has another advantage not mentioned previously. This is one of calibration with respect to cellodextrins. Normally calibration would be carried out by dissolving a carefully weighed amount of dry cellodextrin in a known amount of water and injecting into the LC. However, cellodextrins, prepared in water may be determined by an alternate approach.

An arbitrary amount of cellodextrin, for example G_3, is dissolved in water. The area of the G_3 peak is then determined on the LC (Figure 5 (a)). Next, a known quantity of cellulase enzyme and buffer is added. This mixture is then incubated and the G_3 is completely hydrolyzed to glucose. The glucose concentration is measured on the LC and compared against a glucose standard made from anhydrous reagent grade glucose. The concentration of the G_3 is then back-calculated from the G_1 area on a molar basis (i.e., 1 mole $G_3 \longrightarrow$ 3 moles G_1) and converted to a mass basis. From this technique very accurate response factors are obtained for the cellodextrins (see Table 3). The response factor, which is peak area per unit concentration, is almost the same for the four components shown. As a first approximation then, the LC may be calibrated for soluble cellodextrins using glucose or cellobiose. This is advantageous since glucose and cellobiose, are commercially available, while the other cellodextrins are not.

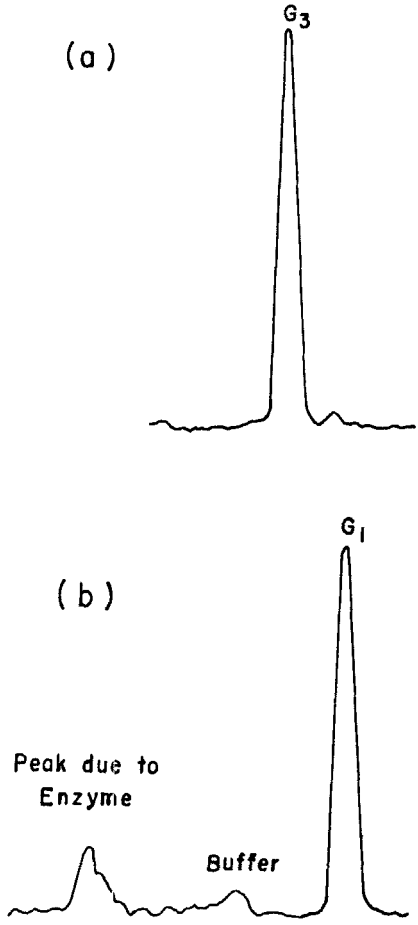

Fig. 5. Complete hydrolysis of G_3 to glucose for calibrating the liquid chromatograph.

The study of the kinetics and mode of action of cellulases consists of incubating a pure enzyme component (either glucanohydrolase, cellobiohydrolase, or cellobiase) with a buffered solution of cellulose or cellodextrins and measuring the products formed. Again, it is desireable to have a method of analysis where (buffer) salts and (enzyme) protein need not be removed prior to analysis. As the examples below will show, aqueous low pressure liquid chromatography is an approach which meets these conditions.

Table 3

Area Response Factors of Pure Cellodextrins Components

Component	Area Units mg/ml
G_1	39.3×10^4
G_2	39.5×10^4
G_3	39.1×10^4
G_4	41.5×10^4

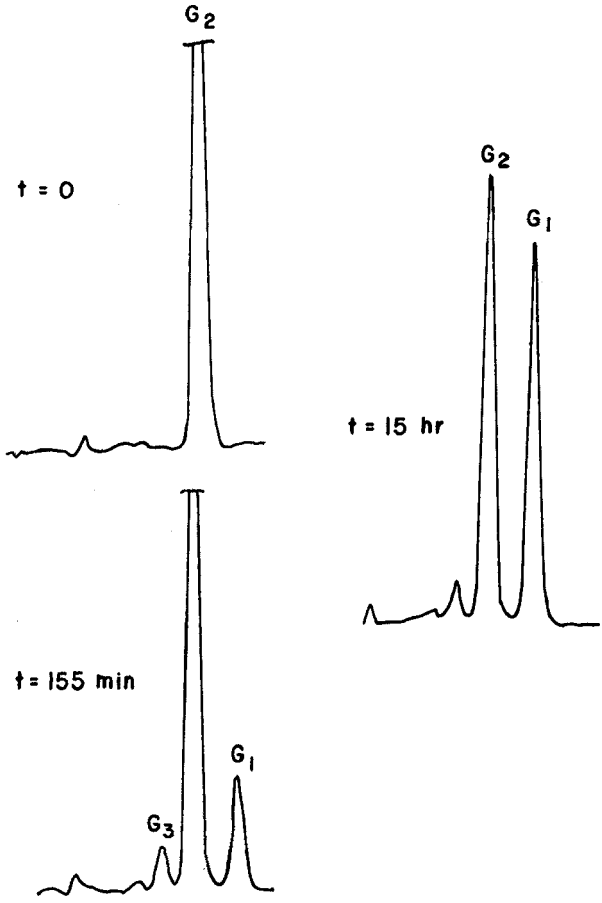

Fig. 6. Cellobiose hydrolysis by glucanohydrolase. Conditions: pH 4.8, 40°C, 17 mM initial cellobiose (G_2); E_T - 1000 µg/ml. (G_3 = cellotriose).

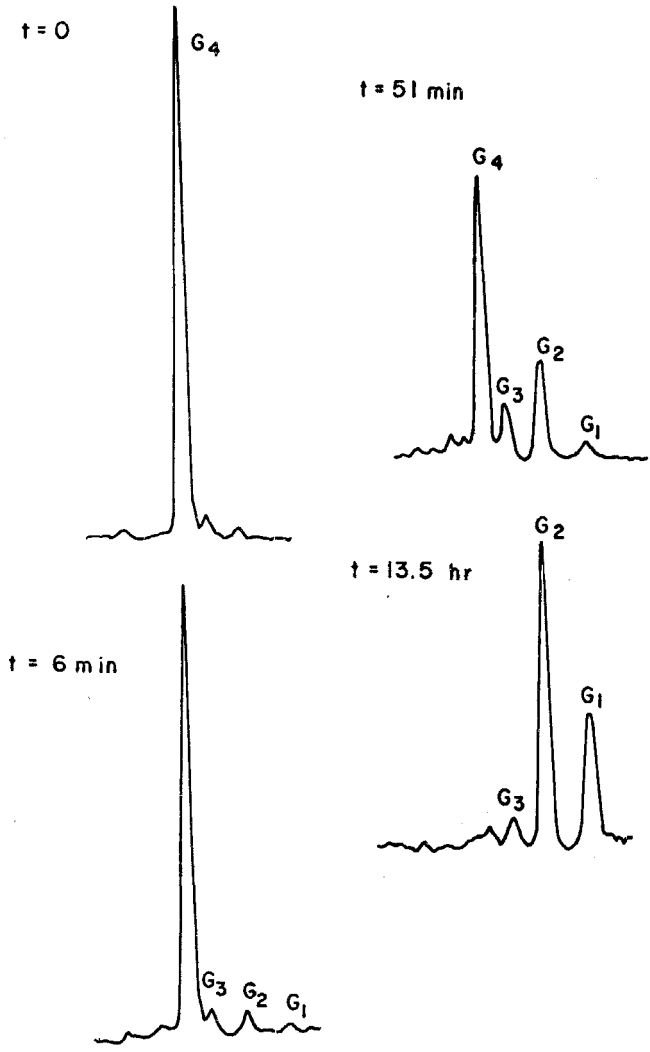

Fig. 7. Timecourse of G_4 (cellotetraose) hydrolysis. Conditions: pH 4.8, 40°C, 4.15 mM initial G_4; E_T = 17.5 µg/ml.

Figure 6 shows the result of incubation of pure glucan-glucanohydrolase (17) with cellobiose. The products are cellotriose, cellobiose, and glucose. A relatively high enzyme concentration (E_T = 1000µg protein/ml) is required to obtain measureable reaction products. The procedure used in this experiment

is to mix enzyme with buffer (sodium acetate, pH 4.8) and substrate to give a total volume of 600 µℓ. This small volume is sufficient since 20 µℓ sample is all that is needed to do an LC analysis.

Figure 7 shows another run made with G_4 and glucanohydrolase. However, here about 50 times less enzyme (E_T = 17.5 µg/ml) was used. It is interesting to note here that the G_4 is totally hydrolyzed without any detectable formation of reversion products.

CONCLUSIONS

Low pressure aqueous liquid chromatography is a useful tool for analysis of cellulolytic activity. It has the advantage of quantitating product distribution as well as total sugars formed. This, together with a short analysis time, small sample size, and a tolerance to salts and proteins, makes the method suitable for analysis of fermentation broths and crude enzyme as well as pure enzymes.

ACKNOWLEDGEMENT

This project was sponsored by the National Science Foundation under AER #7513441. Purdue AES Publication Number 7405.

Presented at the Fourth Joint US/USSR Symposium on Microbial Enzyme Reactions.

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