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HEADSPACE GAS CHROMATOGRAPHIC DETERMINATION OF β-GALACTOSIDASE ACTIVITY USING ELECTRON-CAPTURE DETECTION

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SUMMARY

A headspace gas chromatographic method for the determination of β-galactosidase (E.C. 3.2.1.23) activity is described. The method, in which 2,2,2-trichloroethyl β-D-galactopyranoside (β-TCG) is used as substrate, involves determination of the liberated 2,2,2-trichloroethanol by gas chromatography with electron capture detection. The preparation of β-TCG and of 2,2,2-trichloroethyl α-D-galactopyranoside is described. A $K_m = 0.80$ mM was found for the enzymatic hydrolysis of β-TCG employing β-galactosidase from *Escherichia coli*. The assay has been applied to the quantitative determination of *E. coli* bacteria.

INTRODUCTION

Determination of β-galactosidase activity is widely performed in biochemical and microbiological laboratories, but is also of interest in clinical laboratories in connection with various diseases[1,2] and as part of enzyme immunoassays[3].

β-Galactosidase activity is usually determined by either spectrophotometric[4–6] or spectrofluorimetric[7] methods. The present paper describes a β-galactosidase assay, in which headspace gas chromatography (HSGC) is used for quantifying the enzyme activity.

HSGC has been used in combination with enzyme assays in several cases[8–12] and offers some advantages when compared with the spectrometric methods. The separation of the volatile product from the matrix makes it possible to measure enzyme activities in crude samples, and reduces the time needed for sample preparation.

This assay involves use of the artificial glycoside 2,2,2-trichloroethyl β-D-galactopyranoside (β-TCG) as substrate for β-galactosidase. 2,2,2-Trichloroethanol (TCE), which is liberated during the enzyme reaction, is quantitatively determined by gas chromatography (GC) with electron-capture detection (ECD). The GC determination of TCE is sensitive, and interference from the sample is unlikely because of the selectivity of ECD.

MATERIALS AND METHODS

1,3-Dichloropropan-2-ol (Fluka, Buchs, Switzerland) and 2,2,2-trichloroethanol (Fluka) were dried over calcium hydride and distilled before use.

Sodium dodecyl sulphate (SDS) from Serva (Heidelberg, F.R.G.) and isopropyl β-D-thiogalactoside (IPTG) from Sigma (St. Louis, MO, U.S.A.) were used as received.

β-TCG was synthesized in our laboratory as described below. The substrate was stored in a desiccator below 0°C, and a fresh stock solution was prepared every day by dissolving β-TCG in distilled water.

Solutions of purified β-galactosidase from E. coli were prepared by dissolving lyophilized enzyme (Sigma) in distilled water and adding bovine serum albumin (Sigma) to a final concentration of 0.5 mg/ml.

Raw extract containing α-galactosidase activity was obtained by homogenizing green coffee beans in distilled water (4°C) for 30 min. α-Galactosidase activity was assayed, employing 2,2,2-trichloroethyl α-D-galactopyranoside (α-TCG) as substrate, under conditions similar to those described for β-galactosidase, except that the pH was 6.1 (optimum for α-galactosidase from green coffee beans).

Nuclear magnetic resonance (NMR) spectra were obtained on Jeol FX90Q and Bruker HX-270S spectrometers, and optical rotation was measured on a Perkin-Elmer 141 polarimeter. Preparative HPLC was performed on a Siemens SR100 chromatograph equipped with a Siemens SR210 differential refractometer as detector.

Preparation of substrate

2,2,2-Trichloroethyl 2,3,4,6-tetra-O-acetyl-β-D-galactopyranoside (I) and 2,2,2-trichloroethyl 2,3,4,6-tetra-O-acetyl-α-D-galactopyranoside (II) were prepared as a mixture of anomers according to Magnusson et al.[13]. The anomers were isolated by preparative HPLC using a column (250 mm × 8 mm I.D.) packed with LiChrosorb Si60, 5 μm (Merck, Darmstadt, F.R.G.). The mobile phase was hexane–acetone (3:1); m.p. (I) 108–109°C (109–111°C)[12], m.p. (II) 138–139°C (139–140°C)[12].

2,2,2-Trichloroethyl β-D-galactopyranoside. I (300 mg; 0.6 mmole) was stirred in 10 ml of 0.25 M methanolic sodium methoxide for 2 h at room temperature. The solution was concentrated *in vacuo* to ca. 1 ml and transferred to a column packed with Levatit MP5080 anion-exchange resin (OH$^-$). After elution with distilled water the basic eluates were pooled and treated with a sufficient amount of Amberlite G-50 cation-exchange resin (H$^+$). The neutral solution was freeze-dried leaving 125 mg (65%) of hygroscopic β-TCG; m.p. 162–163°C; $[\alpha]_D^{25} = -12.0°$ (c 1.0, H$_2$O). ^{1}H NMR (270 MHz, ^{2}H$_2$O): δ 3.59–3.95 (6H, m, CHOH) 4.44, 4.55 (2H, 2d, CH$_2$–CCl$_3$, J_{AB} = 12.0 Hz) 4.70 (1H, d, anomeric proton, J = 7.6 Hz).

2,2,2-Trichloroethyl α-D-galactopyranoside. II (300 mg) was hydrolyzed and desalted as described for I. The solution was freeze-dried leaving 155 mg (80%) of hygroscopic α-TCG. No well-defined m.p.; $[\alpha]_D^{25} = +137.5°$ (c 1.0, H$_2$O)). ^{1}H NMR (90 MHz, ^{2}H$_2$O): δ 3.60–4.05 (6H, m, CHOH) 4.19, 4.37 (2H, 2d, CH$_2$–CCl$_3$, J_{AB} = 11.5 Hz) 5.17 (1H, d, anomeric proton, J = 3 Hz).

Bacterial cultures

Suspensions of *E. coli* (strain ML 30) were cultured at 37°C with constant aeration in a buffered solution (pH 7.0) containing 0.1 *M* potassium dihydrogen phosphate, 0.02 *M* ammonium chloride, 0.001 *M* magnesium sulphate 0.01 *M* ferric chloride and with 0.2% sodium succinate solution as the only source of carbon. Exponentially growing cultures were diluted to appropriate concentrations with stock solutions of this medium.

Production of β-galactosidase in the cultures was induced by adding 0.01 *M* IPTG to a final concentration of $1 \cdot 10^{-4}$ *M*. Cultures were induced for 60 min before activity measurements.

The number of colony-forming units (CFU) per ml was determined on standard agar plates (tryptone/glucose/yeast-extract) after incubation for 20 h at 37°C.

Gas chromatography

The HSGC analysis was performed on a Hewlett-Packard 5730A gas chromatograph equipped with a nickel-63 electron capture detector. Chromatograms were recorded on a 10 mV OmniScribe strip-chart recorder (Houston Instruments, TX, U.S.A.). A 27 m × 0.5 mm I.D. support-coated open-tubular glass column GSB/SP1000 (SGE; Ringwood, Australia) was used for the separation. The injection port was maintained at 200°C, the column at 100°C and the detector at 250°C. Nitrogen (purity > 99.9992%) was used as carrier gas (flow-rate *ca.* 10 ml/min) and as make-up gas (flow-rate *ca.* 40 ml/min).

Headspace samples (50 μl) were taken with a 100-μl gas-tight syringe (Hamilton, Bondue, Schwitzerland) and immediately (3–5 s) injected on to the gas chromatograph. To prevent condensation of vapour during the sampling period, the syringe was kept in a thermostated oven at 60°C between injections and removed immediately before sampling.

Assay procedure

A 100-μl portion of substrate solution (25 m*M*), 200 μl of 1 *M* phosphate buffer (pH 7.0) and 100 μl of internal standard (I.S.) solution (1,3-dichloro-propan-2-ol, 13.6 mg/ml) were mixed in a 15-ml glass vial, equilibrated at 37 ± 0.1°C in a thermostated water bath and a 200-μl sample was added. The vial was closed with a silicone-rubber cap and incubated for 60 min. The enzymatic reaction was stopped by injecting 100 μl of 6% SDS solution through the septum with a 1000-μl syringe. After a 30-min equilibration, a 50-μl headspace sample was removed for GC analysis.

RESULTS AND DISCUSSION

A linear relationship between peak-height ratios (TCE/I.S.) and concentration of TCE (0–30 μ*M*) was confirmed by analyzing standards under conditions similar to those described for the enzyme assay. The detection limit (S/N = 2) was 0.3 μ*M*. A similar relationship between peak-height ratios (TCE/I.S.) and enzyme activity has been established for solutions of purified β-galactosidase from *E. coli*. The reproducibility of the enzyme-activity determination was 5.6% (six determinations).

The sensitivity of an enzyme assay is, in principle, limited by the competing

chemical hydrolysis of the substrate. In the present assay, in which β-TCG was used as substrate, there was no measurable hydrolysis at pH 3.5–8.5 when maintaining the substrate solution at 37°C for 20 h. At pH < 3.5, chemical hydrolysis was significant and, therefore, activity measurements in that range should be avoided. In practice, however, the sensitivity was limited by a small amount of free TCE (*ca.* 0.03%) contaminating the substrate, which gave rise to a peak in the chromatogram of the assay blank (Fig. 1). By using an incubation time of 60 min a test-to-blank ratio [peak-height ratio TCE/I.S. (test)/peak height ratio TCE/I.S. (blank)] of 2 corresponds to an enzyme activity of 9 nmol/h/ml of sample. Assay conditions (*e.g.*, pH, substrate concentration) have been chosen to ensure maximum activity when employing β-galactosidase from *E. coli*. The optimum pH was found to be 7.0–7.2. The effect of substrate concentration on enzyme activity is shown in Fig. 2. The maximum activity was found by using substrate concentrations of 4–5 m*M*, while at substrate concentrations of > 5 m*M* inhibition occurred. The K_m was determined from the double reciprocal plot and was found to be 0.80 m*M*.

The maximum rate of hydrolysis (V_{max}), when using β-TCG as substrate, has been compared to the often used o-nitrophenyl β-D-galactopyranoside (ONPG). A ratio V_{max} (β-TCG)/V_{max}(ONPG) $= 0.37$ was found using β-galactosidase, *E. coli*.

β-Galactosidase from *E. coli* is considered as specific for hydrolysing β-galactosides. Similarly, α-galactosidase from green coffee beans is considered specific
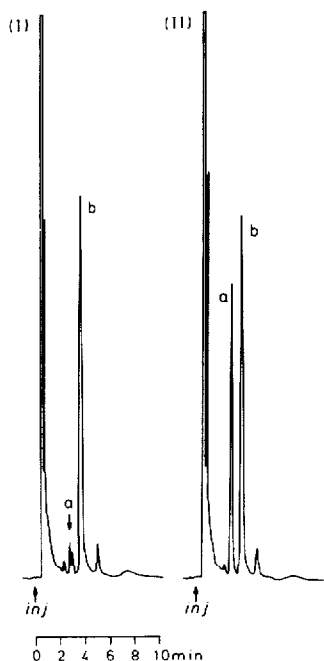

Fig. 1. Typical chromatograms showing I: assay blank (water replacing sample in assay procedure) and II: sample containing *ca.* 2 · 10⁶ *E. coli* bacteria per ml; 50-μl headspace volume injected after incubation for 1 h (a = TCE; b = internal standard). Chromatographic conditions: injection temperature, 200°C; detector temperature, 250°C; column temperature, 100°C. Nitrogen flow-rate, *ca.* 10 ml/min. Attenuation, 8.
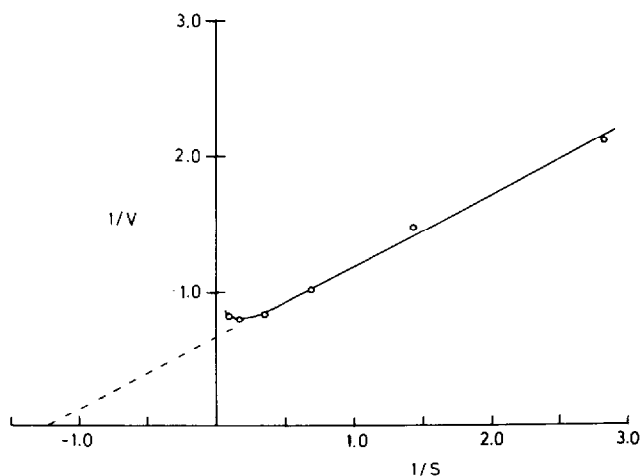
Fig. 2. Double reciprocal plot of the enzymatic hydrolysis of 2,2,2-trichloroethyl β-D-galactopyranoside by β-galactosidase from *E. coli*. S = substrate concentration (m*M*) and V = reaction rate (peak-height ratio: TCE/internal standard). Mean values of duplicates. K_m = 0.80 m*M*.

for α-galactosides. Anomeric specificity of the enzymatic hydrolysis of β-TCG has been examined with those two enzymes. β-TCG and α-TCG were hydrolysed by β-galactosidase and α-galactosidase, respectively, but no detectable enzymatic hydrolysis occurred on incubation of β-TCG with α-galactosidase or of α-TCG with β-galactosidase.

Application of headspace analysis to the GC determination of TCE (b.p. 152°C) has proven useful in several cases[14–16]. In spite of the relatively low volatility of TCE a favourable detector response of the ECD compared to the flame ionization detector (FID) (ratio EC/FID = *ca.* 4 · 10³) has made the headspace analysis possible. The use of an internal standard for the quantitative chromatographic determination was found to be necessary. Reproducibility of the manual headspace sampling was 10–15%, but improved to 3–4% when using an internal standard to correct for the injection volume. 1,3-Dichloropropan-2-ol (b.p. 174°C), which was used as internal standard, must be added in a molar concentration of *ca.* 1000 times the concentration of TCE, primarily because of the weaker response of the ECD.

The low vapour pressure of TCE at 37°C makes the distribution between the gas phase and the liquid phase greatly in favour of the liquid. By choosing an internal standard with a similar characteristic it was possible to remove several headspace samples from the same vial without change in response, provided that the vial was allowed to reach equilibrium between injections. As shown in Fig. 3, an equilibration time of 30 min was found to be sufficient. The possibility of monitoring the enzyme reaction in the gas phase from one sample reduces the time-consuming preliminary experiments always associated with analyzing samples with unknown levels of enzyme activity. For the quantitative determination of enzyme activity, however, termination of the enzymatic reaction by the addition of SDS is necessary to reach equilibrium in the gas phase.

The enzyme assay has been applied to the detection of β-galactosidase activity in induced cultures of *E. coli* bacteria. Since it was found that the presence of internal
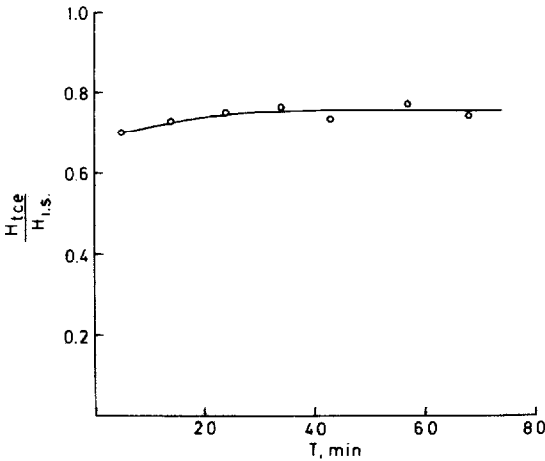
Fig. 3. Effect of equilibration time (min) on headspace analysis of TCE. Ratio of gas-phase concentration of TCE and internal standard measured after incubation at 37°C. Mean values of duplicates.

standard reduced the bacterial rate of multiplication (the generation time increased from 1 h to *ca.* 2 h), the assay procedure was modified for this purpose. Addition of the internal standard was omitted before incubation and was carried out together with the addition of SDS. Furthermore, the phosphate buffer was replaced by the buffered medium described under "Materials and methods" to support the growth of the bacteria during incubation. Fig. 1 shows a typical chromatogram from headspace analysis of an incubated culture of *E. coli*. The relationship between the numbers of *E. coli* bacteria and measured ß-galactosidase activity is shown in Fig. 4. The graph shows a non-linear relationship, which is explained by the continued multiplication of the bacteria during incubation with concomitant production of enzyme. Quantification of *E. coli* bacteria in cultures containing ca. $5 \cdot 10^5 - 1 \cdot 10^7$ CFU/ml was possible after incubation for 1 h by using the described modification of the assay procedure.
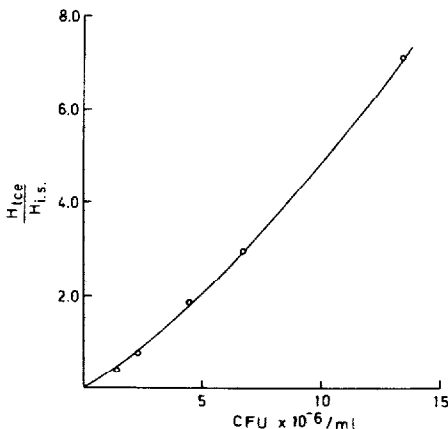

Fig. 4. Activity of β-galactosidase in *E. coli* bacteria as a function of number of colony forming units (CFU) per ml. Activity measured as peak-height ratio TCE/internal standard) following incubation at 37°C for 1 h. Mean values of duplicates.

CONCLUSIONS

The present HSGC assay offers some advantages compared with the frequently used spectrometric methods[4-7] for quantifying β-galactosidase activity. The sensitivity was found to be five to six times that of a standard spectrophotometric method with ONPG as substrate, but is not comparable with the highly sensitive spectrofluorimetric assay[7], which has been used to measure β-galactosidase activities of *ca.* 1 nmol/h/ml sample after incubation for 30 min[1]. However, the combination of volatility and ECD response needed for a determination affords a high degree of selectivity, which makes the headspace analysis directly applicable to crude samples, without interference from the matrix.

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