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Coupling of enzymatic and immunoassay steps to detect *E. coli*: a new, highly sensitive tandem technique for the analysis of low levels of bacteria

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Abstract

A tandem technique for the detection of very low levels *E. coli* within about 2 h is demonstrated. The technique couples the widely employed microbial enzymatic detection methods with an immunoassay step. The bacterial marker enzyme, *E. coli* β-D-galactosidase, was used in conjunction with synthetic enzyme substrates to produce products that could be measured with a highly sensitive enzyme-labelled immunosorbent assay (ELISA). The commercially available 4-methylumbelliferyl-β-D-galactoside and a newly prepared substrate, 4-methylcoumarin-3-propionate-7-O-β-D-galactoside, were used with an ELISA for 7-hydroxy-4-methylcoumarin to demonstrate the detection of low levels of *E. coli*. The 2 h test indicates that a few viable bacteria cells could be detected by the tandem procedure. The end point of the test is an ELISA with colorimetric measurement step. The novel approach retains the essential features of the microbial enzymatic detection procedures and provides a highly sensitive detection system that can be used for rapid screening or quantification of viable microbial cells in water samples. The tandem test is generic for commonly employed glycosidases and other marker enzymes for which 4-methylumbillerone substrates are available.

Keywords: *E. coli*; Immunoassay; β-D-galactosidase; Tandem techniques

1. Introduction

Detection of low levels of bacteria in drinking water and fresh food samples is required for safety and hygiene reasons [1–4]. In this area of public and environmental health, rapid and highly sensitive tests are particularly important due to not only practical and economic pressures but also for public health reasons and the need to take critical decisions quickly in cases of positive results before the consumer is exposed to contaminated water or foods. Both are supplied to the public literally on a continuous basis. Practical and rapid screening methods to eliminate negatives are required by both water monitoring and food safety surveillance agencies [5–7]. The emphasis is on test speed, high sensitivity and accuracy: parameters that are technically contradictory in nature. This is in addition to the normal requirements of simplicity, cost-effectiveness and suitability for high throughput.

The quality of drinking water is an essential element of public health and safety. Yet this vital resource is drawn from rivers, reservoirs and groundwater, which are exposed to many different sources of contamination. Recycling of water for domestic uses and for food processing from wastewater adds to the problems of greater exposure and to more stringent treatment processes [2–4]. Protection of the consumer from microbial infection is heavily regulated by a series of EU Directives [4] as well as national regulations and international agreements (Drinking Water Directives 80/778/EEC and 98/83/EEC and Bathing Water Directive 76/160/EEC). The limits for potentially dangerous microbiological organism such as *E. coli* and faecal coliforms is set at zero in 95% of samples of drinking water, meaning that at least 95% of

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samples tested per annum must have no detectable microbial growth.

A critical element of the suite of such mandatory regulations is the inclusion of enabling analysis technologies adequate to meet the demands of the set regulations. Implementation of the regulations depends entirely on the ability of stakeholders to adopt practical testing regimes able to meet the detection limits and sufficiently practical for frequent applications. In fact, changes in regulations have often been driven by advances in analytical methodologies. One of the several techniques used by water surveillance laboratories is the microbial enzymatic tests with a range of synthetic substrates for microbial marker enzymes [8–14]. Enzymatic methods that take advantages of natural enzymes in microbial living cells and fluorescent or chromogenic enzyme substrates play an important role in the screening of both water and food samples due to relative ease of use, speed, high sensitivity, specificity and availability of a wide range of suitable synthetic substrates. The principal tests currently in use rely on either the detection of glycosidases using fluorimetric substrates or on the metabolism of bacterial ATP to produce bioluminescence [15–18]. The interest in microbial enzymatic detection methods based on naturally occurring microbial glycosidases [19] has been maintained for a long period and is still one of the principal rapid and accurate methods used by surveillance laboratories. A new range of synthetic substrates for glycosidases are described continually to enable achievement of greater sensitivity and convenience [20–25]. The enzymatic detection methods rely on the ability to detect highly fluorescent or chromogenic products (Fig. 1). However, sample matrix interference and optical properties of many hydrolysis products do set a limit as to how far sensitivity can be improved. For example, the excitation and emission wavelengths for the 7-hydroxy-4-methylcoumarins (360 and 460 nm, respectively) are rather low and are therefore prone to interference from complex matrices in addition to requirement of specialised equipment to measure fluorescence.

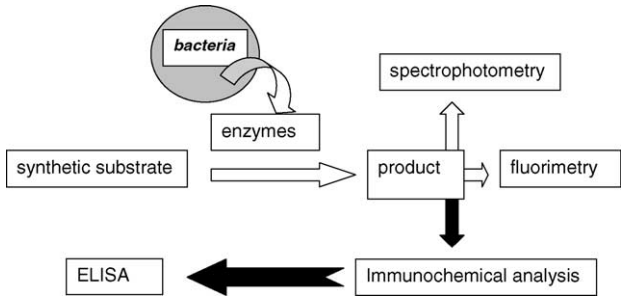

Fig. 1. The element of microbial enzymatic detection methods and end point measurement options. Synthetic substrates for microbial marker enzymes such as glycosidases are selected to provide specificity for marker enzymes and highly detectable products. The proposed scheme is based on measuring the product of the enzyme hydrolysis by an immunoassay in this case enzyme-labelled immunosorbent assay (ELISA).

This report describes a novel approach, in which the enzymatic methods used for the detection of low levels of bacteria (mainly *E. coli* and Coliforms) are further improved by coupling the enzyme-substrate reaction step with an immunological step, in which the product from the hydrolysis of the synthetic substrates is measured by a highly sensitive immunoassay procedure, in this case an enzyme-labelled immunosorbent assay (ELISA). In the scheme, microbial enzyme markers are used to produce "*haptenic*" species or immunologically detectable compounds from synthetic substrates. Hence, according to the analysis scheme the substrates act as masked or chemically protected hapten structures. In this tandem test, the amount of the hapten produced is directly proportional to the levels of the marker microbial enzyme and hence proportional to the levels of viable microbial cells in the test sample.

2. Experimental

2.1. Materials

E. coli O157 culture was from the local laboratory collection. Cultures were grown in Nutrient broth medium and assessed by the viable count procedure on agar plates. Bacterial cultures were serially diluted (10-fold steps) and 1 mL samples were applied to agar plates and after incubation for 24 h the viable count was calculated.

The antiserum to 7-hydroxy-4-methylcoumarin was generated in sheep using a conjugate of 7-hydroxy-4-methylcoumarin-3-acetic acid–bovine serum albumin conjugate as immunogen [26]. Buffer salts, gelatine, 4-methylumbelliferyl-β-D-galactoside (4-MU-gal), goat anti-sheep–horse radish peroxidase (HRP) conjugate, hydrogen peroxide 30% solution, ABTS, 7-hydroxycoumarin-4-acetic acid, acetobromo-α-D-galactose and thin layer chromatography sheets were from Sigma Chemical Company, Pool, UK. 7-Hydroxy-4-methylcoumarin, 7-hydroxylcoumarin-4-acetic acid, resorcinol and diethyl 2-acetylglutarate were from Aldrich Chemical Company, Gillingham, UK. Organic solvents were obtained from BDH, Pool, UK.

2.2. Methods

2.2.1. Preparation of 4-methylcoumarin-3-propionic acid ethyl ester

7-Hydroxy-4-methylcoumarin-3-propionic acid ethyl ester was prepared according to the Pechmann reaction [27] by condensing resorcinol (11 g) and diethyl 2-acetylglutarate (23 g) with concentrated sulphuric acid (25 mL). The reaction was carried out on ice and with due care. The product (Fig. 2) was isolated after pouring the reaction mixture into 2 L of cold water (cooled on ice) and collecting the precipitate by filtration through Whatmman paper using vacuum. The precipitate was dissolved in minimum volume of 0.1 M ice-cold NaOH and re-precipitated by the slow addition of

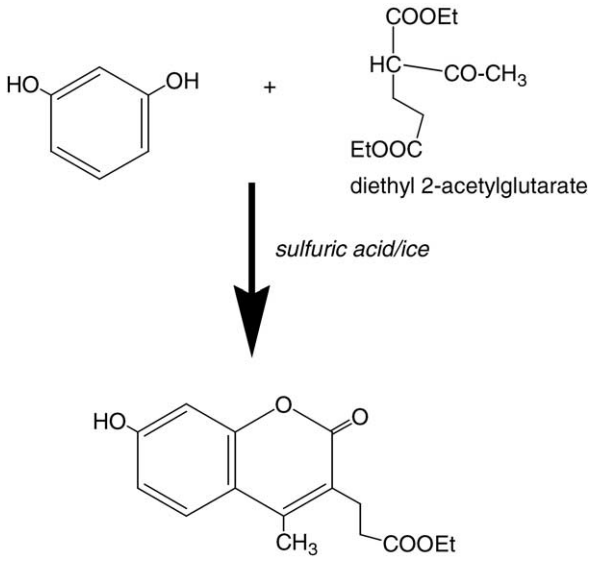

Fig. 2. Synthesis of 7-hydroxy-4-methylcoumarin-3-propionic acid ethyl ester according to the Pechmann reaction.

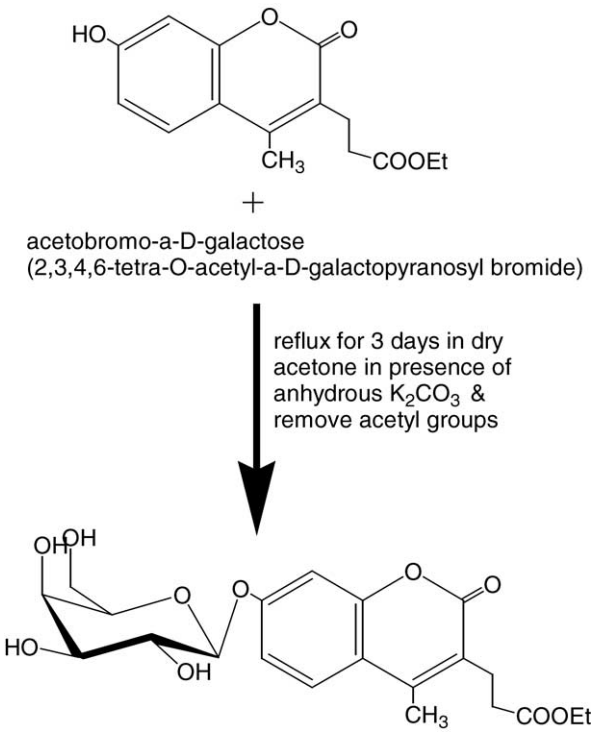

Fig. 3. Synthesis of 4-methylcoumarin-3-propionate-7-O-β-D-galactoside according the Koenings–Knorr reaction.

4 M HCl while stirring. The resultant precipitate was dried (28 g) and dissolved in 300 mL hot ethanol and after the addition of 0.5 mL of concentrated HCl, the mixture was refluxed for 5 h. The product, 7-hydroxy-4-methylcoumarin-3-propionic acid ethyl ester (Fig. 2), crystallised after leaving the mixture to stand in the cold (8 °C). Purity of the highly fluorescent product was established by TLC in three solvent systems.

2.2.2. Preparation of 4-methylcoumarin-3-propionate-7-O-β-D-galactoside

This galactoside substrate was prepared according to Koenigs–Knorr reaction [28] by refluxing for 3 days a mixture of 7-hydroxy-4-methylcoumarin-3-propionate (0.5 g) and acetobromo-α-D-galactose (2.5 g) in dry acetone (45 mL) in the presence of anhydrous potassium carbonate (1 g). Formation of the acetylated galactoside was followed by TLC in chloroform:methanol (90:10). Identification of spots was carried out by inspection under UV light where the product had greatly reduced fluorescence and stained positive for sugars after spraying with sulphuric acid (12% in methanol) and heating. The acetylated galactoside was taken up (after removal of acetone) in chloroform (250 mL) and extracted with cold 0.5 M NaOH till all traces of the starting materials were removed from the chloroform layer. The dried chloroform was removed and de-acetylation was carried out by addition of a few drops of 5% methanolic KOH to the product dissolved in minimum volume of methanol. The substrate (Fig. 3), 4-methylcoumarin-3-propionate-7-O-β-D-galactoside, was precipitated by the addition of diethyl ether.

2.2.3. Enzyme-immunoassay for 7-hydroxy-4-methylcoumarin and 7-hydroxy-4-methylcoumarin-3-proionic acid ester

The solid phase competitive enzyme immunoassay (ELISA) for the measurement of 7-hydroxy-4-methylcoumarin and 7-hydroxy-4-methylcoumarin-3-proionic acid ester was carried out by mixing the coumarin derivative with the antibody (1/20,000) for about 30 min followed by bringing the mixture (150 μL aliquots) into contact with the competitor attached to surface of microtitre plate wells (7-hydroxylcoumarin-4-acetic acid–gelatine conjugate, coating with 150 μL of 10 μg/mL) and allowed to mix for 20 min (to bind free antibodies). The fraction of antibodies bound to solid phase was measured using goat anti-sheep–HRP conjugate (1/2000, 1 h at 37 °C). The colour was developed using H_2O_2 and ABTS (40 μL and 50 mg, respectively per 100 mL of 50 mM sodium acetate–citrate buffer, pH 4.1) and measured at 410 nm (Anthos 2000 plate reader).

ELISA calibration graphs of reference ligand, 7-hydroxy-4-methylcoumarin and 7-hydroxy-4-methylcoumarin were constructed using series of known concentrations of the substances. Estimation of the cross-reactivity of galactoside substrates was carried by the 50% inhibition test.

2.2.4. Detection of E. coli

In this procedure, the naturally occurring *E. coli* β-D-glycosidase was employed to hydrolyse the synthetic substrate to produce the antigenic ligand, 7-hydroxy-

4-methylcoumarin or 7-hydroxy-4-methylcoumarin-3-proionic acid ester depending on substrate used. The produced ligand was measured by the ELISA step.

E. coli from cultures of known viable counts suspended in 1 mL of PBS containing 0.5% sodium dodecyl sulphate (SDS), the galactoside substrate (1 mL of 100 μg/mL in 0.1 M sodium phosphate buffer, pH 7.4, 0.1 M NaCl, 10 μM $MgCl_2$ and 0.2% gelatine) and the antibody (100 μL of 1/100 in the same buffer) were shaken for about 1 h before aliquots of 150 μL were transferred to coated wells of assay microtitre plates and the ELISA steps were continued as above.

E. coli cells trapped on 0.45 μ filters were estimated by dipping the filters in buffer and continuing as above.

3. Results and discussion

According the scheme in Fig. 1, *E. coli* β-D-galactosidase hydrolyses the glycoside substrate to produce aglycon moiety of the substrate which is then measured by a competitive ELISA. This essentially means that the greater the number of *E. coli* cells, the more of aglycon is produced and the higher will the inhibition in the competitive ELISA step. It follows that the test signal is inhibition of binding of the antibody in the ELISA step compared with binding in the absence of any bacteria. Two substrates were used in the study: a commercial 4-MU-β-D-galactoside and 4-methylcoumarin-3-propionate-7-O-β-D-galactoside preparation (Fig. 4). The hydrolysis products from the substrates (may be seen here as haptens) are immunochemically different in terms of response in the competitive ELISA. 7-Hydroxy-4-methylcouarin is a hapten without the linkage arm whereas the 7-hydroxy-4-methylcoumarin-3-propionic acid ester is a full hapten consisting of the coumarin nucleus and the linkage arm, which adds considerable binding affinity toward the antibody. The greater affinity toward the antibody enabled the full hapten to be detected in greatly

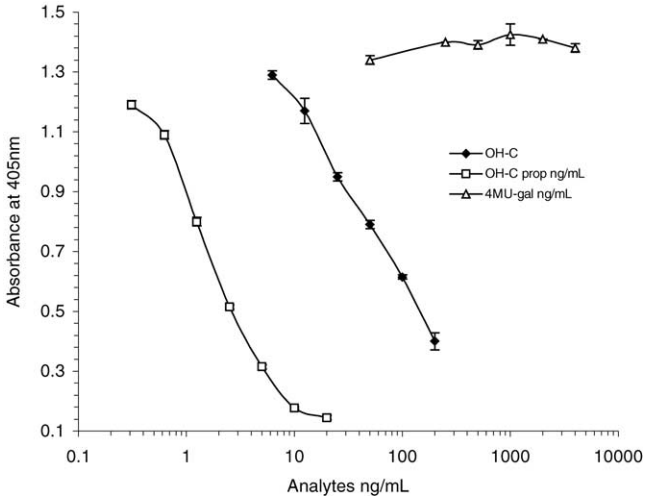

Fig. 5. ELISA calibration graphs for 7-hydrox-4-methylcoumarin (OH–C) and 7-hydroxy-4-methylcoumarin-3-propionic acid ethyl ester (OH–C prop) and the cross-reaction level of 4-MU-gal. The response to OH–C prop is much greater than to OH–C due to higher affinity toward the antibody. The cross-reaction level of 4-MU-gal is effectively zero.

reduced concentrations (by comparison with 7-hydroxy-4-methylcouarin), which in turn meant that less bacteria could be detected when this substrate was used.

The initial phase of the study involved constructing the ELISA procedure using pure reference standards of the detectable aglycons and demonstrating that the substrates do not interfere with the ELISA step. For the substrate not to cause significant interference, they should have little or no cross-reactivity toward the antibody. Dose response graphs using the described competitive ELISA indicated minimum detectable levels of 1 ng/mL for the full hapten and about 10 ng/mL for 7-hydroxy-4-methylcoumarin (Fig. 5). The cross-reaction of the substrate was below the detection limits of the test, virtually no cross-reactivity was observed with the quantities tested (Fig. 5).

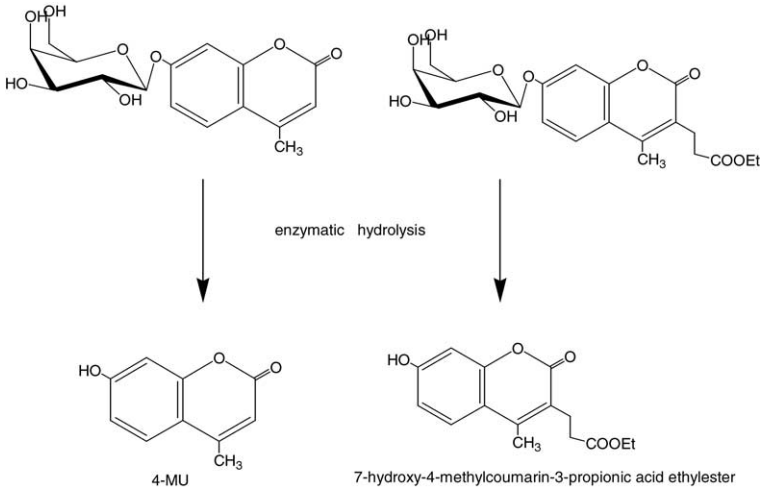

Fig. 4. Hydrolysis of 4-MU-β-D-galactoside (Sigma substrate) and 4-methylcoumarin-3-propionate-7-O-β-D-galactoside.

The sensitivity by which *E. coli* could be detected was demonstrated using cell cultures of known viable count values. Cells, substrate solution and antibody were mixed where the release of aglycon and binding to antibody were expected to occur take place during this step. The transfer of aliquots of the reaction mixture to coated assay wells initiated the competitive ELISA phase. The ELISA response to increasing levels of bacteria was as expected in accordance with competitive immunoassay in which the signal is inversely proportional to the analyte or the bacterial count. Typical ELISA response to varied levels of bacteria, 10–10^8 cells/mL is shown in Fig. 6. The figure shows two graphs, the ELISA responses using Sigma 4-MU-gal substrate (graph A) and curve B using 4-methylcoumarin-3-propionate-7-O-β-D-galactoside (own preparation). The sensitivity of the curves is completely different. The dynamic range of the test shown by curve A appears to be between 10^5–10^{10} cell/mL whereas curve B indicates a range of detection between 10 and 10^5 cells/mL. The result in principle was expected but the magnitude of difference in detection sensitivity given by the two substrates perhaps was not. Since the test conditions were identical except for the substrates, it is concluded that the difference is due to the affinity of the antibody toward the detectable hapten released by the enzyme. The probably easier access of the propionic acid ester substrate to cellular enzyme due to its structure (propionic acid ethyl ester arm) or higher affinity of the enzyme toward this substrate may also be contributory factors. The higher affinity of antibody toward the full hapten is demonstrated in the calibration graph given in Fig. 5 and clearly shown by the greatly reduced minimum detection limit.

The results provide evidence that it is entirely possible to detect a few cells of *E. coli* by the described 2 h tandem assay provided that the cells are trapped into a small sample volume.

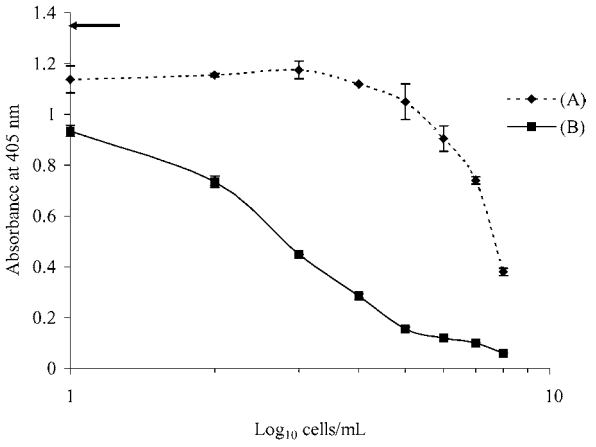

Fig. 6. Detection of *E coli* by the tandem enzymatic-immunochemical technique described in this report using 4-MU-gal (curve A) and 4-methylcoumarin-3-propionate-7-O-β-D-galactoside (curve B). The response curves for increasing levels of cells (10–10^8 cells/mL) demonstrate the principle of the method and clearly show the increased sensitivity given by the second substrate (own preparation). The response (absorbance at 405 nm) is the final competitive ELISA signal and hence appears inversely proportional to the levels of *E. coli*. The arrow shows the absorbance value in the absence of bacteria (100% signal).

The tandem assay couples two enzymatically driven steps: the microbial enzyme hydrolysis and the enzyme immunoassay steps. The detectable antigen (hapten) for the immunoassay step is produced by enzymatic hydrolysis of a substrate or a masked hapten structure. Thus, both steps in the tandem method involve signal bio-amplification by virtue of enzyme catalysis of substrates which are not present in limited amounts and therefore the potential sensitivity and speed of the test can be expected to be high, as shown in this preliminary study. The 2 h test can be used for the detection of a few *E. coli* cells, perhaps single cells under fully optimised test conditions, with a colorimetric end point and without interference with the essential features of the accepted enzymatic methods for microbial detection.

For the test to operate the masked (protected) hapten, structure must meet a number of critical conditions including: stability under test conditions, has very low or preferably no cross-reactivity toward the antibody and display favorable kinetic properties to hydrolysing by the enzyme marker. 4-Methylcoumarin-3-propionate-7-O-β-D-galactoside meets these conditions to varying but mainly acceptable degrees. The results emphasise the importance of the immunochemical element of the tandem technique and clearly point toward areas where further technically feasible improvements can be made in order to achieve detection limits compatible with the requirements of water and food rapid analysis. Both assay sensitivity and speed can be improved significantly by using haptens with higher affinity and by decreasing test incubation steps.

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