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Note: Cyclohexenoesculetin-β-D-glucoside: a new substrate for the detection of bacterial β-D-glucosidase

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A.L. JAMES, J.D. PERRY, M. FORD, L. ARMSTRONG AND F.K. GOULD. 1997. A new substrate for the detection of bacterial β-D-glucosidase was evaluated as an alternative to aesculin. This substrate, 3,4-cyclohexenoesculetin-7-β-D-glucoside, was compared with aesculin for the detection of β-D-glucosidase in 150 enterococci, 40 streptococci, 12 *Listeria* sp. and 250 strains of Enterobacteriaceae. In the Gram-positive strains tested, aesculin hydrolysis correlated with hydrolysis of 3,4-cyclohexenoesculetin-7-β-D-glucoside. In the Gram-negative strains the new substrate was hydrolysed by all aesculin-positive strains and also by four strains (10%) of *Escherichia coli* which gave a negative aesculin reaction. 3,4-Cyclohexenoesculetin-7-β-D-glucoside was shown to be a reliable alternative to aesculin and was shown to have significant advantages over aesculin when incorporated into solid media. This was due to the non-diffusible end product produced by hydrolysis of 3,4-cyclohexenoesculetin-7-β-D-glucoside in the presence of iron.

INTRODUCTION

The presence of β-D-glucosidase has long been regarded as an important taxonomic marker in microbial identification. The most commonly used substrate for the detection of this enzyme is the naturally occurring glycoside aesculin. Hydrolysis of aesculin yields β-D-glucose and esculetin (6,7-dihydroxycoumarin), the latter compound being detected by the formation of a brown/black complex in the presence of iron salts. This test was first applied in the identification of enterococci and has since found wide application in the identification of other genera (Swan 1954; Wasilauskas 1971). The main disadvantage of this substrate is that, when incorporated in agar, the resulting complex formed spreads throughout the medium (James and Yeoman 1987). This creates difficulties in distinguishing β-glucosidase-producing colonies when present within a mixed culture. The spreading of the esculetin complex is not reduced significantly by increasing the concentration of agar.

Synthetic substrates are also available for detection of β-D-glucosidase to yield either chromogenic or fluorescent compounds upon hydrolysis (Manafi *et al.* 1991). For example, the fluorogenic compound 4-methylumbelliferyl-β-D-glucoside has been widely used although it has several disadvantages when incorporated into an agar medium. These include the fact that the recognition of colonies can only be performed under the presence of long-wave u.v. light and the released fluoriphore tends to diffuse throughout the agar. Other substrates commonly employed include β-D-glucoside derivatives of nitrophenol (Trepeta and Edberg 1987); however, widespread diffusion is again a severely limiting factor when incorporating such substrates into solid media (Manafi *et al.* 1991).

Due to the limitations of these substrates, chromogenic compounds have been employed which yield insoluble products upon hydrolysis. Such substrates provide the advantage that the released chromogen remains localized around the bacterial colony without diffusing through the medium (Kodaka *et al.* 1995). Examples of these include indoxyl-β-D-glucoside and 5-bromo-4-chloro-3-indolyl-β-D-glucoside (James 1994). Whilst these substrates are highly effective they are relatively difficult to prepare and although commercially available, their extremely high cost has proved prohibitive for large scale diagnostic use. The use of another novel compound, 8-hydroxyquinoline-β-D-glucoside, has been described as an alternative to aesculin (James and Yeoman 1987). Although impressive results were obtained, toxicity

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problems have been encountered particularly with Gram-positive organisms (Albert *et al.* 1953).

We describe a new compound, 3,4-cyclohexenoesculetin-7-β-D-glucoside as a proposed alternative to aesculin. The structure of this compound and product of hydrolysis by β-D-glucosidase are shown in Fig. 1. We evaluate the effectiveness of this compound in direct comparison with aesculin for a wide range of enterococci and Enterobacteriaceae strains.

MATERIALS AND METHODS

Aesculin and ferric ammonium citrate were obtained from Sigma Chemicals Company Ltd, Poole, UK. Columbia agar was obtained from Lab M, Bury, UK. Chemicals involved in the synthesis of 3,4-cyclohexenoesculetin-7-β-D-glucoside were obtained from the Aldrich Chemical Company Ltd, Gillingham, UK.

3,4-Cyclohexenoesculetin-7-β-D-glucoside (CHE-β-D-glucoside) was prepared as follows: 3,4-cyclohexenoesculetin was prepared by a standard Pechmann reaction involving condensation of ethyl 2-cyclohexanonecarboxylate with 1,2,4-triacetoxybenzene in the presence of 75% w/w sulphuric acid. Typical batch yields were between 80 and 90%. Preparation of the β-glucoside was effected by performing a modified Koenigs–Knorr reaction as follows: 3,4-cyclohexenoesculetin was dissolved in an excess of 10% potassium hydroxide solution. To this was added an equimolar proportion of α-acetobromoglucose dissolved in acetone. The homogeneous solution was stirred overnight and poured into an ice/water mixture. The solid phase which separated was collected on a filter and dissolved in dichloromethane and this solution was washed well with water. The dried organic layer was then evaporated to give a grey solid. This was dissolved in hot ethanol and subsequently recrystallized to yield the 7-*O*-tetra-acetyl glucoside of 3,4-cyclohexenoesculetin. The dry product was dissolved in methanol and treated with a catalytic amount of sodium methoxide. After several hours thin layer chromatography confirmed complete deacetylation. The pH of the resulting solution was reduced to around 6·5 by addition of an aqueous slurry of an ion-exchange resin (*IR:120 H+*) to the well stirred solution and then rapidly decanting. Methanol was removed by rotary evaporation under reduced pressure until crystallization commenced. The glycoside produced was redissolved in hot aqueous methanol and recrystallized to give a white powder.

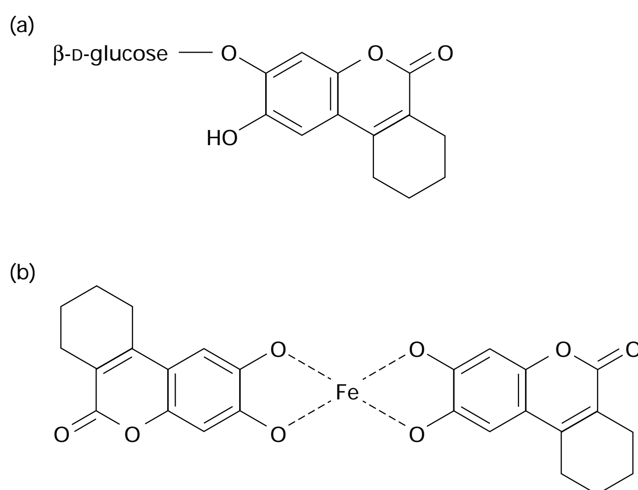

Fig. 1 Structure of cyclohexenoesculetin-β-D-glucoside and the complex formed following hydrolysis. (a) 3,4-Cyclohexenoesculetin-β-D-glucoside; (b) cyclohexenoesculetin molecules released by hydrolysis associate with iron to form a black insoluble complex

Aesculin agar was prepared as follows: 41 g of Columbia agar, 0·5 g ferric ammonium citrate and 1·0 g of aesculin were dissolved by boiling in 1 l of distilled water. The pH of this medium was then adjusted to 7·5 and the medium sterilized by autoclaving for 10 min at 116°C. The medium was then allowed to cool to 55°C before being poured in 20 ml volumes. CHE-β-D-glucoside agar was prepared in an identical fashion except that 0·5 g of CHE-β-D-glucoside was substituted for aesculin.

One hundred and fifty strains of enterococci collected from a wide range of clinical and environmental samples were identified using the scheme of Facklam and Collins (1989). Each strain was inoculated into physiological saline to produce a suspension equivalent to McFarlane standard 0·5 ($\approx 10^8$ organisms ml^{-1}). Using a multipoint inoculator (Denley), 1 μl of each suspension was inoculated onto both of the test media. Twelve strains were inoculated per plate. In addition to the enterococci a selection of 40 streptococci and 12 strains of *Listeria* sp. were collected and identified with the API 32 STREP (BioMérieux) with complimentary tests where indicated. These were also inoculated in identical fashion. Two hundred and fifty strains of Enterobacteriaceae collected from a wide range of sources were identified by the API 20E system (BioMérieux). These were inoculated onto both aesculin and CHE-β-D-glucoside media as described above.

Finally, seven strains of enterococci and two strains of streptococci were obtained from the National Collection of Type Cultures (NCTC), London. These were *Enterococcus faecium* NCTC 7171, *Enterococcus faecalis* NCTC 755, *Enterococcus gallinarum* NCTC 11428, *Enterococcus mundtii* NCTC 12343, *Enterococcus casseliflavus* NCTC 12341, *Enterococcus durans* NCTC 8307, *Enterococcus raffinosus* NCTC 12192, *Streptococcus agalactiae* NCTC 8181 and *Streptococcus bovis* NCTC 8177. Suspensions of these nine strains were prepared as above at 10^8 organisms ml^{-1} and diluted in sterile distilled water by standard methods to produce suspensions of approximately one organism per ml. Three × 100 ml volumes of each suspension were then filtered onto three cellulose nitrate membrane filters (Sartorius) using a standard

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filtration technique. The filters were placed onto a CHE-β-D-glucoside plate, an aesculin plate and a Columbia agar plate without substrate as a growth control. All plates were incubated at 37 °C in air for exactly 18 h, except for the plates inoculated with streptococci which were incubated in air supplemented with 5% carbon dioxide. After incubation plates were examined for the presence of black or brown colonies and colony counts were performed on the membrane filters.

RESULTS

All strains used in this study grew well on both CHE-β-D-glucoside and aesculin-containing media. The two substrates

Table 1 Hydrolysis of aesculin and cyclohexenoesculetin (CHE)-glucoside by a variety of bacterial species

Species	No. of strains	CHE-glucoside (% positive)	Aesculin (% positive)
Gram-positive			
Enterococcus casseliflavus	1	100	100
Ent. durans	2	100	100
Ent. faecalis	83	100	100
Ent. gallinarum	3	100	100
Ent. raffinosus	5	100	100
Ent. faecium	56	100	100
Listeria ivanovii	2	100	100
L. monocytogenes	10	100	100
Streptococcus agalactiae	7	0	0
Strep. bovis	10	100	100
Strep. dysgalactiae	2	0	0
Strep. mitis	4	0	0
Strep. mutans	2	100	100
Strep. oralis	4	0	0
Strep. pneumoniae	4	0	0
Strep. pyogenes	2	100	100
Strep. sanguis	5	40	40
Gram-negative			
Citrobacter diversus	5	100	100
Cit. freundii	11	0	0
Enterobacter aerogenes	2	100	100
E. agglomerans	1	100	100
E. cloacae	20	80	80
Escherichia coli	40	10	0
Escherichia hermanii	1	0	0
Hafnia alvei	7	0	0
Klebsiella oxytoca	20	100	100
Kl. ozaenae	1	100	100
Kl. pneumoniae	35	100	100
Proteus mirabilis	15	0	0
Pr. penneri	2	0	0
Pr. vulgaris	5	100	100
Providencia rettgeri	2	50	50
P. stuartii	7	0	0
Salmonella sp.	21	0	0
Serratia liquefaciens	17	100	100
Ser. marcescens	20	100	100
Shigella flexneri	2	0	0
Sh. sonnei	9	0	0
Yersinia enterocolitica	4	0	0
Y. pseudotuberculosis	3	100	100

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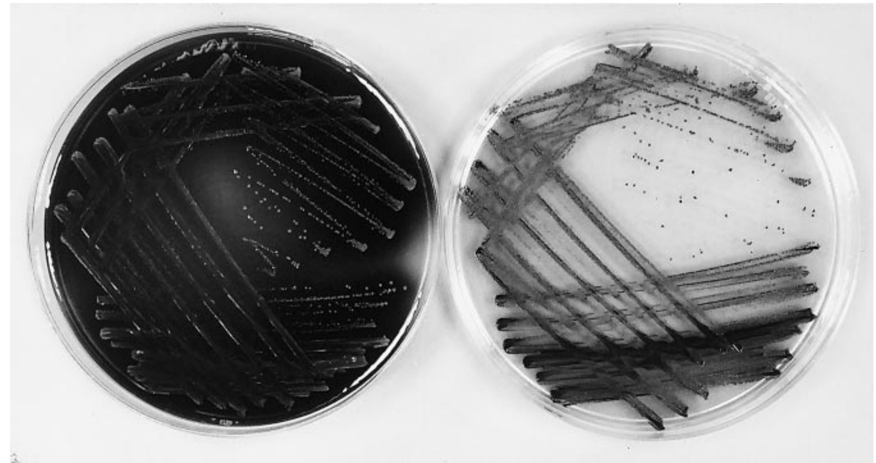

Fig. 2 *Listeria monocytogenes* (NCTC 11994) demonstrating β-D-glucosidase activity on CHE-β-D-glucoside (right) and a traditional aesculin agar (left)

were markedly different, however, with respect to the diffusion of the product through agar following hydrolysis. Hydrolysis of aesculin resulted in a black complex which diffused widely whereas hydrolysis of CHE-β-D-glucoside produced a black complex highly restricted to the bacterial growth. It can be seen from Table 1 that the hydrolysis of CHE-β-D-glucoside correlated extremely closely with hydrolysis of aesculin. This was particularly true of the Gram-positive bacteria where all 150 strains of enterococci and 12 strains of *Listeria* sp. hydrolysed both aesculin and CHE-β-D-glucoside to produce black colonies. Excellent correlation was also achieved between these two substrates for the Gram-negative bacteria tested. The exception to this was in the case of *Escherichia coli* where four strains (10%) appeared to hydrolyse CHE-β-D-glucoside but were unable to hydrolyse aesculin

In the membrane filtration experiment all NCTC strains of enterococci and the strain of *Streptococcus bovis* produced black colonies on CHE-β-D-glucoside medium. On the filters placed on aesculin-containing medium, all of the strains produced a light brown diffusible complex which spread across the surface of the filter and also into the agar below the membrane. There was no statistical difference between the colony counts on CHE-β-D-glucoside, aesculin or Columbia agar for any of the organisms tested (data not shown). *Streptococcus agalactiae* NCTC 8181 served as a negative control and did not demonstrate hydrolysis of either substrate.

DISCUSSION

The main advantage of CHE-β-D-glucoside is that when the released cyclohexenoesculetin combines with iron, the resulting black complex is highly insoluble and does not diffuse through the agar medium. This results in the formation of discrete black colonies which can easily be differentiated within mixed cultures. In contrast when aesculin is hydrolysed, the released esculetin combines with iron to form a brown/black complex which diffuses rapidly though the agar. This diffusion can lead to difficulties in distinguishing aesculin-positive colonies within a mixed culture. The difference between the two substrates is highlighted in Fig. 2. The absence of any diffusion of the black complex when using CHE-β-D-glucoside might also provide a considerable advantage when identifying bacteria using a multipoint inoculation technique if large numbers of strains are applied to each plate.

Another highly useful attribute of CHE-β-D-glucoside which has emerged from this study is its potential application in membrane filtration studies for faecal streptococci/enterococci. Aesculin has long been regarded as a potentially useful substrate for such a purpose; however, the problems associated with the diffusion of the esculetin complex have not been overcome despite much effort (Daoust and Litsky 1975). This problem of diffusion is particularly acute when large numbers of enterococci are present and the whole membrane is stained with the tan-coloured esculetin complex (Brodsky and Schiemann 1976). When CHE-β-D-glucoside is used as the substrate, enterococci grow as discrete black colonies on the membrane which can be easily differentiated within a mixed population. This is because the black complex formed within the colony is unable to diffuse through the membrane and into the agar, hence any colour produced is highly restricted to the actual colony. In the case of aesculin, the smaller complex formed readily diffuses through the membrane into the agar and the brown colouration produced is poorly visible due to widespread diffusion. This difference between the two substrates is demonstrated in Fig. 3.

It has been reported that hydrolysis of aesculin may be rapidly detected by monitoring the disappearance of the natural substrate fluorescence, during hydrolysis in the presence of iron salts (Edberg *et al.* 1976). CHE-β-D-glucoside, unlike aesculin, is not fluorescent due to substitution of the 7-hydroxyl group of the cyclohexenoesculetin molecule.

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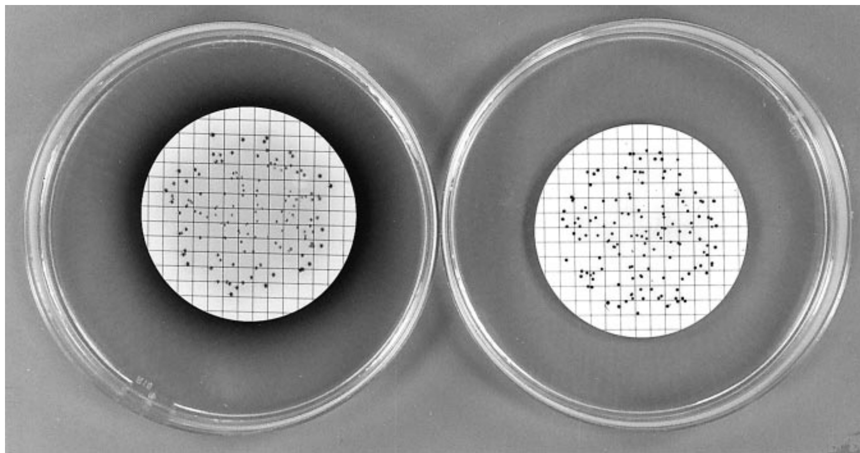

Fig. 3 *Enterococcus faecalis* (NCTC 755) demonstrating β-D-glucosidase activity on membrane filters on CHE-β-D-glucoside medium (right) and a traditional aesculin agar (left)

However, when this substrate is hydrolysed the cyclohexenoesculetin released is fluorescent. Therefore, an alternative strategy for a fluorescence assay using CHE-β-D-glucoside would be to look for generation of fluorescence in the absence of iron salts. In conclusion, CHE-β-D-glucoside is a highly useful non-inhibitory substrate which produces a black non-diffusible product upon hydrolysis by β-D-glucosidase in the presence of iron. It is clear that CHE-β-D-glucoside will be a more expensive compound than the widely available aesculin. However, the cost of the raw materials and the ease of its synthesis should ensure that CHE-β-D-glucoside is less expensive than many commercially available alternatives such as the indoxyl derivatives.

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