SHORT ARTICLES

INHIBITION OF DIFFERENT SEROTYPES OF LISTERIA MONOCYTOGENES BY ENTEROCINS IN SOLID AND LIQUID MEDIA

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The production of bacteriocins (enterocins) is a very common property of enterococci (Sherwood et al., 1949; Stark, 1960; Brock, Peacher and Pierson, 1963; Brandis, Brandis and Van De Loo, 1965; Pleceas, 1970; Tzannetis, Leonardopoulos and Papavassiliou, 1970; Brandis and Smarda, 1971; Bottone, Allerhand and Pisano, 1974). Two bacteriocins, enterocin E1A and E1B, produced by Streptococcus faecium strain E1 and studied in detail (Krämer and Brandis, 1975a and b) acted not only against certain strains of enterococci and Streptococcus salivarius but also inhibited strains of Clostridium perfringens and Clostridium septicum (Krämer and Schallehn, 1974) and strains of Listeria monocytogenes in solid media (Brandis and Brandis, 1962; Krämer and Brandis, 1975a). This paper describes the production of enterocins by several strains of different species of enterococci in solid and liquid media and the action of these inhibitory substances on different serotypes of L. monocytogenes.

MATERIALS AND METHODS

Cultures. All strains of bacteria tested were isolated from human sources. The organisms were grown in tryptose phosphate broth (TPB, Difco). Strains of enterococci were classified as Streptococcus faecium, Streptococcus faecalis, Streptococcus faecalis v. zymogenes or Streptococcus faecalis v. liquefaciens (Heeschen, Tolle and Zeidler, 1967; Esper, 1970). Isolates of L. monocytogenes were identified by biochemical methods and further subdivided into the serotypes 1, 2, 3, 4a, 4b, 4c, 4d and 4e.

Demonstration of enterocin activity

Stab plate method. A modification of the method of Fredericq (1948) was used. Cultures of enterococci were stabbed into TPB agar plates, incubated for 18 h, and after exposure to chloroform vapour were overlaid with 3.0 ml of soft TPB agar containing approximately 10^7 cells of the indicator strain per ml. Inhibition zones around the stab culture after incubation for 18 h at 37°C indicated enterocin sensitivity.

Spot test. Single drops of purified enterocin E1A (see below) or of TPB culture supernatant fluids, the latter filtered through Millipore membranes (Millipore, London) of 0.22 µm porosity, or heated at 80°C for 15 min., were spotted on to TPB agar containing 10^7 cells of the indicator strain per ml. The plates were examined for inhibition of growth after incubation for 18 h at 37°C.

Enterocin titre. Serial dilutions of supernatant fluids of cultures of enterococci were incubated with equal volumes of exponential-phase broth cultures of the indicator strain containing 10^4 bacteria per ml. The reciprocal of the highest dilution that completely inhibited growth after incubation for 8 h at 37°C was recorded as the titre of the sample.

Production and purification of enterocin E1A. Enterocin E1A, found without induction in the supernatant fluid of cultures of S. faecium strain E1, was purified 400-fold by consecutive ammonium sulphate fractionation, gel filtration on Sephadex G-75 and chromatography on DEAE-Cellulose (Krämer and Brandis, 1975a).
TABLE I

Demonstration of enterocins in solid and liquid cultures

<table>
<thead>
<tr>
<th>Enterocin-producing streptococcus</th>
<th>Number of strains showing inhibitory activity in solid medium</th>
<th>Number of strains showing inhibitory activity in supernate of fluid culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of strains tested</td>
<td>Heated</td>
</tr>
<tr>
<td>S. faecium</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>S. faecalis</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>S. faecalis v. zymogenes</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>S. faecalis v. liquefaciens</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>

TABLE II

Inhibition of Listeria monocytogenes by enterococcal cultures in solid media

<table>
<thead>
<tr>
<th>Donor streptococcal strain</th>
<th>Number of sensitive L. monocytogenes strains of the stated serotype (and number of strains tested)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 (22)</td>
</tr>
<tr>
<td>S. faecium strain no. E1 3 25 38 44 59 77</td>
<td>21</td>
</tr>
<tr>
<td>S. faecalis strains no. 8, 61, 68, 98</td>
<td>20</td>
</tr>
<tr>
<td>S. faecalis v. zymogenes strain no. 24 78 81 85</td>
<td>21</td>
</tr>
<tr>
<td>S. faecalis v. liquefaciens strains no. 4, 5, 12, 66, 75</td>
<td>20</td>
</tr>
</tbody>
</table>

Lethal effect of enterocin E1A. Indicator cells from exponential phase cultures in TPB were centrifuged, washed and resuspended in fresh medium. Purified enterocin E1A or, as a control, 0.06 M-phosphate buffer, were added and the mixtures incubated aerobically at 37°C. The number of surviving viable cells was determined at intervals thereafter by subculturing diluted 0.1-ml samples on to TPB agar.

RESULTS

Demonstration of soluble enterocins in liquid cultures of enterococci

Twenty-one of the strains of different species of group D streptococci found by Brandis et al. (1965) to produce at least one enterocin when grown on solid media were examined for their production of soluble enterocins in liquid cultures. The strains tested are listed in table II. Enterocins were detected in the supernatant fluid of cultures of 14 of them (table I).
**Enterocins Active Against Listeria**

**Table III**

Susceptibility of *Listeria monocytogenes* serotypes to soluble enterocins of *Streptococcus faecium*

<table>
<thead>
<tr>
<th>Enterocin-producing <em>S. faecium</em> (strain no.)</th>
<th>Titre of enterocin inhibitory action against <em>L. monocytogenes</em> of serotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>E1</td>
<td>320</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
</tr>
<tr>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>38</td>
<td>160</td>
</tr>
<tr>
<td>44</td>
<td>80</td>
</tr>
<tr>
<td>59</td>
<td>320</td>
</tr>
<tr>
<td>77</td>
<td>10</td>
</tr>
</tbody>
</table>

Soluble enterocins of *S. faecium* were heat stable but significantly adsorbed to membrane filters whereas the enterocins produced by *S. faecalis, S. faecalis v. zymogenes* and *S. faecalis v. liquefaciens* were completely inactivated by heating for 15 min. at 80°C.

**Inhibition of *Listeria monocytogenes* in solid media**

When the susceptibility to enterocins of *L. monocytogenes* was tested by the stab plate method, the zones of inhibition produced were of different sizes depending on the enterocinogenic strain and the strain of *L. monocytogenes* under test. As shown in Table II, enterocin activity was species-specific. All the strains tested except *L. monocytogenes* serotype 4c were susceptible to the enterocins of three or more strains of *S. faecium*. However, four only of 51 strains of *L. monocytogenes* were inhibited by cultures of other species of enterococci.

**Inhibition of *Listeria monocytogenes* in liquid media**

There was a noticeable variation in the sensitivity of different serotypes of *L. monocytogenes* to the action of enterocins from different strains of *S. faecium* (Table III). Two serotypes (4c and 4e) were completely resistant; 4 serotypes (1, 3, 4a and 4d) were inhibited by all but one of the soluble enterocins tested and two serotypes (2 and 4b) were of intermediate sensitivity.

**Killing effect on sensitive cells**

*S. faecium* strains E1, 38 and 59 caused the most marked inhibition of the greatest number of strains of *L. monocytogenes*. It has been shown (Krämer and Brandis, 1975a and b) that the inhibitory substance produced by *S. faecium* strain E1 can be separated into two distinct bacteriocins—enterocin E1A and E1B. The figure shows the lethal action of purified enterocin E1A at different concentrations on sensitive cells of *L. monocytogenes* serotype 1, as demonstrated by loss of colony forming units. Purified enterocin E1A with a titre of 150 killed over 99% of the indicator cells in 10 min. at 37°C. The optical densities of the cell suspensions with and without enterocin E1A did not show any appreciable difference during incubation for 1 h.

**Discussion**

Bacteriocins are defined as antibacterial substances produced by various species of bacteria which are active usually against closely related micro-organisms; however, some activity on species outside the bacteriocinogenic group is common, especially for bacteriocins produced by Gram-positive bacteria (Hamon and Peron, 1963). Apparently, most bacteriocins produced by *S. faecium* and by a few strains of other enterococci are of the type active also against some unrelated genera. Thus, most of the 51 strains of *L. monocytogenes*...
tested were found to be sensitive to at least one bacteriocin produced by different strains of enterococci. The inhibition of *L. monocytogenes* could be demonstrated as easily on agar medium as in liquid cultures. There were, however, great differences in the spectrum of activity of the bacteriocins according to the species of the producer strain. The bacteriocins of *S. faecium* had the widest range and inhibited 50 strains of *L. monocytogenes*, whilst relatively feeble activity against *L. monocytogenes* was shown by bacteriocins of *S. faecalis*, *S. faecalis* v. *zymogenes* and *S. faecalis* v. *liquefaciens*. Activity of *S. faecium* bacteriocins has been demonstrated also against different strains of enterococci (Krämmer and Brandis, 1975a) and against strains of *C. septicum* (Krämmer and Schallehn, 1974), and other workers have also demonstrated a broad spectrum of activity for bacteriocins from *S. faecium* (Pieceaș, Bogdan and Vereanu, 1971), *S. faecalis* v. *zymogenes* (Brock et al., 1963; Bottone et al., 1974) and other enterococci (Pohunek, 1961).

Brandis et al. (1965) demonstrated that bacteriocins of *S. faecium* were inactivated by the action of trypsin in solid media, whereas bacteriocins of *S. faecalis*, *S. faecalis* v. *zymogenes* and *S. faecalis* v. *liquefaciens* were resistant to trypsin. Furthermore, the present study shows that these bacteriocins vary not only in their spectrum of activity but also in their response.
to membrane filtration and to heat; the bacteriocins produced by strains of \textit{S. faecium} differ clearly in these two respects from those produced by other enterococci.

The action of purified \textit{S. faecium} enterocin E1A on \textit{L. monocytogenes} appeared to be primarily bactericidal but despite a rapid decrease in the number of viable cells of \textit{L. monocytogenes} after the addition of enterocin E1A there was no striking reduction in optical density of the cell suspension. These results suggest that the lethal effect was not accompanied by significant cell lysis as demonstrated for other enterocins which are both strongly haemolytic and strongly bacteriolytic (Davie and Brock, 1966; Jackson, 1971). Bactericidal action with no reduction in optical density has also been observed when enterocin-sensitive cells of \textit{S. faecium} were used instead of \textit{L. monocytogenes} (Kramer and Brandis, 1975b) and after the addition of a bacteriocin of \textit{S. faecalis} v. \textit{zymogenes} to a sensitive strain of \textit{C. perfringens} (Botto et al., 1974).

**SUMMARY**

Twenty-one enterocinogenic strains of enterococci were examined for their ability to inhibit 51 strains of \textit{Listeria monocytogenes} belonging to eight different serotypes; 50 strains of \textit{L. monocytogenes} were uniformly inhibited on solid media and in broth cultures by strains of \textit{Streptococcus faecium}. However, only four strains of \textit{L. monocytogenes} were inhibited by strains of \textit{Streptococcus faecalis}, \textit{S. faecalis} v. \textit{zymogenes} or \textit{S. faecalis} v. \textit{Zyquefaciens}. Enterocin E1A prepared from the supernatant fluid of \textit{S. faecium} strain E1 rapidly killed sensitive cells of \textit{L. monocytogenes} but did not lyse them.

I wish to thank Mrs Christel Reichertz for her excellent technical assistance.

**REFERENCES**


