Purification and Characterization of a Bacteriocin from *Klebsiella pneumoniae* 158

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Klebocin, a bacteriocin produced by *Klebsiella pneumoniae* 158, was purified to homogeneity by ammonium sulphate fractionation and sequential DEAE-Sephacel and Sephadex G-150 column chromatography. The purified preparation had an *M*, of approximately 40000 on SDS-PAGE. Chemical analysis of the purified preparation showed it to be a protein, and it was sensitive to digestion by various proteolytic enzymes.

**INTRODUCTION**

Interest during the last decade in klebocins, which are bacteriocins produced by *Klebsiella* species, can be attributed to their use as epidemiological markers for typing strains; this technique is simpler and more reproducible than other typing methods (Buffenmeyer _et al._, 1976; Chugh _et al._, 1979, 1982; Sabherwal _et al._, 1983). However, no attempts have been made to purify klebocins, and consequently little information is available on their physico-chemical nature. This study was undertaken to isolate, purify and characterize the klebocin produced by *Klebsiella pneumoniae* 158.

**METHODS**

*Bacteria. * *Klebsiella pneumoniae* 158, a klebocin producer, and *Klebsiella pneumoniae* WC, the indicator strain, were procured from the Medical College, Rohtak, India.

**Preparation of crude klebocin.** The inoculum of the producer strain was prepared in Trypticase Soy Broth (TSB) by growing the organism for 16 h at 30 °C on a gyrorotatory shaker (180 r.p.m.). A 10 ml volume of this seed culture was added to 50 ml fresh TSB and incubated for 1 h at 30 °C on the shaker. Mitomycin C was then added to give a concentration of 0.5 μg ml⁻¹ and incubation was continued for 8 h. Then 1 ml chloroform was added to the medium and cell-free supernate was prepared by centrifugation (8000 g, 15 min, 4 °C). This supernate represented the crude klebocin.

**Assay of klebocin.** Serial twofold dilutions of the sample were prepared with Tris/HCl buffer (0.05 M, pH 7.4). A lawn of 8 h growth of *K. pneumoniae* WC (indicator strain) was made on a nutrient agar plate by spreading 100 μl of the culture. A 20 μl sample of each dilution of test solution was spotted onto the lawn. The spots were allowed to dry at room temperature and the plates were incubated for 12-14 h at 32 °C. The reciprocal of the highest dilution showing complete inhibition of the indicator strain represented the klebocin titre.

**Purification of klebocin.** Cell-free supernate (1450 ml) was made 70% saturated with solid ammonium sulphate by adding small amounts with constant stirring, and stored at 4 °C for 12 h. The precipitate was removed by centrifugation (8000 g, 20 min, 4 °C), dissolved in 24 ml Tris/HCl buffer (0.05 M, pH 7.4) and dialysed against 2-litre volumes of the same buffer for 12 h at 4 °C with several changes. Particulate matter was removed by centrifugation (4000 g, 10 min, 4 °C), and 10 ml of the supernate was applied to a DEAE-Sephacel column (2.6 x 12 cm). The initial elution (2 bed volumes) was done with Tris/HCl buffer (0.05 M, pH 7.4) followed by a stepwise gradient of sodium chloride (0.1 - 0.3 M). The active fractions were pooled, concentrated by lyophilization, dialysed against Tris/HCl buffer (0.05 M, pH 7.4) for 6 h and chromatographed on a Sephadex G-150 column (1.6 x 70 cm) using Tris/HCl buffer (0.05 M, pH 7.4) as eluent. The active fractions were collected and concentrated by lyophilization.

**Electrophoresis.** To monitor the purification, each fraction was electrophoresed on 7.5% (w/v) polyacrylamide gels (Laemmli, 1970). The gels were stained with Coomassie blue, thoroughly destained and scanned at 595 nm on a Gilford spectrophotometer 250. SDS-PAGE was used for determining the *M*, (Weber & Osborn, 1969).
Chemical analysis. Purified klebocin was assayed for protein (Lowry method), carbohydrate (Dubois et al., 1956), total lipid (Friggs & Dunn, 1970), phosphorus (Bartlett, 1959), DNA (Burton, 1956) and RNA (Herbert & Phipps, 1971).

Enzyme treatment. Purified klebocin (titre 64 units) was incubated separately with the following enzymes (each at 0.5 mg ml⁻¹) for 1 h at 37 °C: trypsin (bovine pancreas, type III), chymotrypsin (bovine pancreas, type IV), pronase B, protease (from Streptomyces griseus, type XIV), ribonuclease (bovine pancreas, type I-AS) and lysozyme (egg white, grade I).

Chemicals and media. Enzymes except pronase B (Calbiochem) were from Sigma. Mitomycin C was from Kyowa Hakko Kogyo Ltd. TSB and nutrient agar were from Hindustan Dehydrated Media. A standard protein kit for M₃ determination was purchased from Pharmacia.

RESULTS

The ammonium sulphate precipitated klebocin preparation showed a fourfold increase in specific activity of klebocin. DEAE-Sephalac chromatography of the ammonium sulphate precipitate showed four well-defined peaks (Fig. 1(a)). The klebocin activity was observed in peak 1 which was eluted with buffer in the absence of sodium chloride. Material eluting in this peak was concentrated and chromatographed on Sephadex G-150 (Fig. 1b). Two components were separated; most of the klebocin activity was concentrated in the first peak which was sharp and symmetrical. The klebocin activity followed the same pattern as that of protein concentration. Table 1 summarizes the purification. The final preparation showed a 19-fold increase in specific activity and the yield was 1.3%. The purified lyophilized preparation was
Table 1. Activity and recovery of klebocin at each step of purification

<table>
<thead>
<tr>
<th>Test material</th>
<th>Volume (ml)</th>
<th>Protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific activity [units (mg protein)^{-1}]</th>
<th>Recovery (%)</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-free supernate</td>
<td>1450</td>
<td>2465</td>
<td>185600</td>
<td>75.3</td>
<td>100</td>
<td>–</td>
</tr>
<tr>
<td>(NH_4)_2SO_4 (70% sat.) precipitate dissolved in 0.05 M-Tris/HCl pH 7.4</td>
<td>24</td>
<td>768</td>
<td>24576</td>
<td>320</td>
<td>13.2</td>
<td>4.2</td>
</tr>
<tr>
<td>DEAE-Sephacel peak 1</td>
<td>25</td>
<td>7.2</td>
<td>6400</td>
<td>888.9</td>
<td>3.4</td>
<td>11.8</td>
</tr>
<tr>
<td>Sephadex G-150 peak 1</td>
<td>2.0</td>
<td>1.8</td>
<td>2560</td>
<td>1422.2</td>
<td>1.3</td>
<td>19.0</td>
</tr>
</tbody>
</table>

Fig. 2. Electrophoretic pattern of purified klebocin in a 7.5% polyacrylamide gel stained with Coomassie blue.

Purified klebocin was not conjugated with lipid, carbohydrate or phosphorus compounds, as none of these could be detected in the preparation. A small amount of RNA was detected which could have been either a contaminant or part of an RNA–protein complex. Itoh et al. (1978) also detected a small amount of RNA (7.3 µg ml^{-1}) in their purified preparation of carotovoricin Er, a bacteriocin of Erwinia carotovora; they suggested that this, and the small amount of carbohydrate detected in the preparation, were possibly due to contamination by cell envelopes or ribosomes.

The klebocin activity of the purified preparation was completely destroyed by trypsin, chymotrypsin, pronase B and protease but was not affected by ribonuclease or lysozyme.

DISCUSSION

Klebocin from Klebsiella pneumoniae 158 was purified to homogeneity. To our knowledge this is the first report on the purification of a klebocin. The purified preparation had an $M_r$ of approximately 40000. The $M_r$ values of bacteriocins vary considerably: 27000–92000 for colicins (Herschman & Helinski, 1967; Kunugita & Matasuhashi, 1970; Schwartz & Helinski, 1971; Timmis, 1972; Braun et al., 1974); 72000–95000 for pyocins (Sano & Kageyama, 1981); 60000 for cloacin DF13 (De Graaf et al., 1970); 64000 for marcescin (Foulds, 1972); and 65000 for pesticin A-122 (Hu & Brubaker, 1974).

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The klebocin activity was completely destroyed by different proteolytic enzymes. The RNA fraction did not appear to be involved in the activity as treatment with ribonuclease was not inhibitory. Colicin K-K235 and bacteriocin of Proteus morganii strain MR336 are lipoglycoprotein (Goebel & Barry, 1958) and glycoprotein (Smit et al., 1968), respectively, but the activity of these two bacteriocins resides exclusively in the protein moiety, as observed in this study.

REFERENCES


