Purification of a Mucopolysaccharidase from \textit{Bacteroides distasonis}

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A mucopolysaccharidase derived from a pathogenic strain of \textit{Bacteroides distasonis} was isolated and purified by fractionation with cold acetone and ion-exchange chromatography on DEAE-cellulose, pH 8.0. Three detectable enzyme activities from concentrated supernatant filtrates were obtained in a fraction precipitated by three volumes of cold acetone; these were DNAase, hyaluronidase and chondroitinase-like activity. Separation of the DNAase was achieved by ion-exchange chromatography. Fractions designated as purified mucopolysaccharidase contained both hyaluronidase and chondroitinase-like activity.

\section*{INTRODUCTION}

The role of the members of the genus \textit{Bacteroides} as agents of disease has recently been appreciated more fully. These non-sporeforming anaerobic bacilli are the most common cause of anaerobic infection in man and animals (Finegold et al., 1971; Finegold & Rosenblatt, 1973; Levinson, 1973). Characteristically the lesions produced are deep-seated, foul-smelling abscesses. Local extension, especially in soft tissues, bacteremia and metastatic abscess formation are common sequelae to such abscesses (Rein & Cosman, 1971; Walker & Wilkins, 1976; Willis et al., 1974).

The mechanism by which such spreading lesions are produced has yet to be ascertained. It has been reported (Rudek & Haque, 1976) that two mucopolysaccharidases, hyaluronidase and chondroitinase, are produced by certain clinically significant species of the genus. Salyers et al. (1977) also reported the presence of such enzymes while studying the fermentation of various mucopolysaccharides by Bacteroides. More recently, Steffen \textit{et al.} (1979) demonstrated these enzymes from those Bacteroides most frequently isolated from clinical specimens. The role of such enzymes in facilitating the spread of an infectious agent and thus enhancing virulence has been postulated for other organisms (Sallman & Birleland, 1950). Such a mechanism may also play a role in some \textit{Bacteroides} infections.

The purpose of the present investigation was to purify the mucopolysaccharidases from a strain of \textit{Bacteroides distasonis}. Purified fractions would not only be useful in characterizing these enzymes but also in determining their role in the infectious process.

\section*{METHODS}

\textbf{Organism.} A strain of \textit{Bacteroides distasonis} isolated from a patient at the University of Illinois Hospital was studied. This culture was chosen since it exhibited large zones of hydrolysis on hyaluronate substrate agar plates. The organism also produced three other detectable enzymes \textit{in vitro}. These were a chondroitinase-like activity, DNAase and an alkaline phosphatase.

\textbf{Culture medium.} The basic growth medium for enzyme production was Brain Heart Infusion broth (BHI, Difco), with yeast extract (Difco) added to a final concentration of 1\% (w/v). Immediately after heat

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sterilization the medium was placed in an anaerobic glove box (model AL1250, Coy Manufacturing Co., Ann Arbor, Mich., U.S.A.), cooled, and inoculated with a single entire colony of B. distasonis, as well as some of the underlying agar, by means of a sterile Pasteur pipette. The broth culture was incubated anaerobically for 72 h at 37°C. The cells were removed by centrifugation and filtration.

**Concentration of cell-free filtrate.** Lyophilization and ultrafiltration (Sober et al., 1956) were used to concentrate the filtrate 20- to 150-fold. These concentrates were used for qualitative and quantitative determination of enzyme activity. Protein concentration was determined by the Lowry method.

**Fractionation of cell-free filtrates.** Concentrated cell-free filtrate was fractionated by precipitation with 3 vol. chilled acetone (−20°C). (This was the lowest ratio of acetone which resulted in complete recovery of mucopolysaccharidase activity in the precipitate.) The mixture was maintained at 4°C for 30 min. The precipitate was then recovered by centrifugation at 6000 g for 15 min and dissolved in 1-0 ml sterile distilled water.

**DEAE-cellulose chromatography.** A column (0.9 × 30 cm) packed with DEAE-cellulose (Whatman, DE 52) was equilibrated with 0.005 m-sodium phosphate buffer, pH 8.0. The acetone precipitate, equilibrated in similar buffer, was then applied to the column. The column was washed with this starting buffer and eluted by increasing the NaCl concentration (0.05 to 0.5 M) in batch gradations. The flow rate was 70 ml h⁻¹.

**Gel electrophoresis.** The method of Ornstein (1964) was used for discontinuous gel electrophoresis. The buffer was Tris/glycine, pH 8.4 (containing 6 g Tris 1⁻ and 28.8 g glycine 1⁻). The concentration of the acrylamide gel was 8% (w/v). Samples containing 50 or 150 µg protein (purified and crude fractions, respectively) were layered in a 40% (w/v) sucrose solution on top of the sample gel, and then a current of 5 mA per tube was passed for 1 h. A method for electrophoretic localization was also used: by comparing the patterns of the gels stained with Coomassie blue (0.2%, W/V) with unstained gels placed on substrate agar plates, it was possible to determine which stained bands corresponded to various enzymic activities.

**Assay of enzyme activities.** Hyaluronidase was quantitatively measured by a modified mucin clot prevention (MCP) method (Murphy, 1972) and by a turbidity-reducing unit (TRU) method (Kass & Seastone, 1944). The MCP method involved the incubation of 50 µl hyaluronic acid with 25 µl of test material or 25 µl of serially diluted test material in separate wells. After incubation at 37°C for 20 min followed by refrigeration at 4°C for 30 min, 25 µl 2 M-acetic acid was added. Prevention of mucin clot formation indicated hyaluronidase activity. The highest dilution of test material which prevented formation of a mucin clot was recorded.

The TRU method was used to determine activity in assessing the degree of purification. For this, 0-2 ml of a hyaluronic acid stock solution was incubated for 45 min at 38°C with an equal volume of test material; acid albumin (2-0 ml) was then added and the absorbance at 600 nm was determined. Hyaluronidase activity was estimated from the decrease in the turbidity of the solution. Units of activity were determined from a standard curve.

Substrate agar plate methods were also used to determine hyaluronidase and chondroitinase-like activity (Smith & Willett, 1968), DNAase activity (Porshen & Sonntag, 1974) and phosphatase activity (Porshen & Spaulding, 1974).

**RESULTS**

**Detection of enzyme activities**

Hyaluronidase and chondroitinase-like activity were always found together in all fractions tested. Pooled samples were assayed quantitatively for hyaluronidase activity by the MCP method. MCP titres of 1:1 to 1:32 were found in crude filtrates depending on the broth medium used for growth. Concentrated cell-free filtrates from ultrafiltration and acetone precipitation had MCP titres of 1:256. Turbidimetric reduction techniques indicated a similar ratio of activity between the crude filtrate and the acetone precipitates (5-6 units and 56 units, respectively).

In addition to the mucopolysaccharidases only one other enzyme activity, DNAase, was detected in the cell-free filtrates. Phosphatase activity, though detectable in cultures growing on agar plates, did not appear in the extracellular filtrates.

**Fractionation by ion-exchange chromatography**

Treatment of the concentrated cell-free filtrate with 3 vol. chilled acetone resulted in complete recovery of the DNAase and mucopolysaccharidase activities in the precipitate. This precipitate was chromatographed on DEAE-cellulose, pH 8.0 (Fig. 1). Since only
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Fig. 1. DEAE-cellulose chromatography (pH 8.0) of the acetone precipitate. The hyaluronidase and chondroitinase-like activity (HAase) and DNAase activity of the fractions were detected by the substrate agar plate method. The details of the ion exchange are given in Methods.

Table 1. Steps in the purification of a mucopolysaccharidase from Bacteroides distasonis

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein concn (mg ml⁻¹)</th>
<th>Activity (TRU ml⁻¹)</th>
<th>Specific activity (TRU mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude filtrate</td>
<td>3.66</td>
<td>5.6</td>
<td>1.53</td>
</tr>
<tr>
<td>Acetone ppt.</td>
<td>3.82</td>
<td>56.0</td>
<td>14.6</td>
</tr>
<tr>
<td>Pooled fractions from DEAE-cellulose, pH 8.0</td>
<td>0.09</td>
<td>11.3</td>
<td>125.5</td>
</tr>
</tbody>
</table>

TRU, Turbidity-reducing units.

three enzyme activities were detectable in the acetone precipitates, i.e. DNAase, hyaluronidase and chondroitinase, the ion-exchange chromatography conditions were chosen to exclude the DNAase activity from certain fractions. On DEAE-cellulose, the DNAase was eluted as one minor and one major peak at fractions 7 to 9 and 12 to 16, respectively. The mucopolysaccharidases were eluted as three minor peaks and one major peak. The major peak (fractions 24 to 28), which was devoid of DNAase activity, was designated as purified mucopolysaccharidase. Table 1 summarizes these purification steps.

Gel electrophoresis

Discontinuous gel electrophoresis of the concentrated cell-free filtrates resulted in multiple proteinaceous bands. After acetone precipitation, only three bands were evident. Following DEAE-cellulose chromatography, the pooled major peak (fractions 24 to 28) exhibited only two bands, approximately 12 and 14 mm from the origin. Electrophoretic localization using unstained gels demonstrated that, in the acetone precipitates, three enzyme activities were present: one at the origin corresponded to DNAase and the other two at 12 to 14 mm corresponded to the mucopolysaccharidase. The pooled fractions (24 to 28) from the DEAE-cellulose column when electrophoresed showed enzyme activity only in one area, 12 to 14 mm from the origin.

DISCUSSION

The purification of this mucopolysaccharidase is a step towards understanding the role of such enzymes in the infectious process. The nature of the lesions produced by Bacteroides,
typically deep-seated abscesses with local extension and metastatic abscess formation (Willis et al., 1974), points to a role for some extracellular component. Studies in this area have led to suggestions that phosphatase (Porshen & Spaulding, 1974), DNAase (Porshen & Sonntag, 1974), heparinase (Felnerr & Dowell, 1971), collagenase (Gibbons & MacDonald, 1961) and fibrinolysin (Weiss, 1937) may be mediators in the diseases caused by this group. With the exception of collagenase, however, none of the other extracellular enzymes appear to be responsible for the spreading type of lesion. Collagenase production, although implicated in pathogenesis, has only been demonstrated in one species, B. melaninogenicus. It thus provides no explanation for the pathogenesis of the other Bacteroides, especially the frequently isolated members.

Our previous finding of two such hydrolases (Rudek & Haque, 1976), namely hyaluronidase and chondroitinase, in Bacteroides is thus of considerable interest. Since these enzymes are produced by some of the clinically significant species of Bacteroides, as demonstrated by Steffan et al. (1979), it is possible that both may play a role in the pathogenesis of this group.

To study these enzymes in a cell-free environment the organism was grown in a broth medium. In this medium, the level and number of enzyme activities was decreased. Despite extreme concentration of the crude filtrates, only three of the four extracellular enzymes shown to be produced by the culture on substrate agar plates could be demonstrated in cell-free filtrates. Phosphatase activity could not be demonstrated, although it was detected when B. distasonis cells were tested. This is in agreement with the observation of Cheng & Costerton (1973) that phosphatase activity in B. ruminicola and B. succinogenes is located exclusively in the periplasmic space of exponential phase cells. This would explain why only three detectable enzyme activities remained in the cell-free filtrates.

Although DNAase activity could easily be excluded by altering the charge on the DEAE-cellulose at pH 8.0, the mucopolysaccharidases always remained together. The fractions designated as purified mucopolysaccharidase contained both hyaluronidase and chondroitinase-like activity. Other investigators have reported similar findings in other systems. Hahn (1947) using the most highly purified testicular hyaluronidase preparations then available still reported that they were able to break down chondroitin sulphate. More recently, Thurston et al. (1975) have shown that hyaluronic acid as well as a number of other mucopolysaccharides are susceptible to degradation by a purified chondroitinase from Proteus vulgaris. Since the hydrolysis of hyaluronic acid and chondroitin sulphate is brought about by breakage of the (1→4)-β linkage, both mucopolysaccharides may be cleaved by a single enzyme. However, since our disc electrophoresis experiments resulted in the isolation of two bands, the possibility exists that in this organism we may be dealing with two closely complexed enzymes.
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