Subcellular Localization of D-Glucanases in *Bacteroides oralis* Ig4a

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Three D-glucan-hydrolysing enzymes from *Bacteroides oralis* Ig4a have been isolated. Two of them are dextranases which hydrolyse (1→6) but not (1→3) linked α -D-glucans; one (EC 3.2.1.11, 1,6- α -D-glucan 6-glucanohydrolase) is localized in the periplasm, and the other, which is an exo-enzyme (EC 3.2.1.70, 1,6- α -D-glucan glucohydrolase), in the cytoplasm. The third is a mutanase (EC 3.2.1.59, 1,3-(1,3;1,4)- α -D-glucan 3-glucanohydrolase) that hydrolyses (1→3) but not (1→6) linked α -D-glucans, and is present only in the cytoplasm.

INTRODUCTION

Extracellular polysaccharides, especially glucans, are considered to be important matrix substances in dental plaque (Gibbons & Nygaard, 1968; Guggenheim & Schroeder, 1967) and are thought to participate in the initiation of dental caries (Fitzgerald & Jordan, 1968; Gibbons & Banghart, 1967; Newbrun, 1976). D-Glucanases such as dextranase (EC 3.2.1.11, 1,6- α -D-glucan 6-glucanohydrolase or EC 3.2.1.70, 1,6- α -D-glucan glucohydrolase) and mutanase (EC 3.2.1.59, 1,3-(1,3;1,4)- α -D-glucan 3-glucanohydrolase) have been tested by a number of workers with the aim of eliminating the accumulation of dental plaque. Dextranase (Caldwell *et al.*, 1971; König & Guggenheim, 1968; Nyman *et al.*, 1972) and mutanase (Guggenheim *et al.*, 1972; Kelstrup *et al.*, 1973; Simonson *et al.*, 1983) were less effective in eliminating dental plaque when applied separately. The combined application of both enzymes has been reported to be effective in eliminating mature dental plaque *in vivo* (Guggenheim & Haller, 1972; Staat *et al.*, 1982; Takahashi *et al.*, 1982). We have found that a strain of *Bacteroides oralis* Ig4a produces both dextranases and a mutanase and the present study was undertaken to determine the subcellular localization of these enzymes.

METHODS

Culture medium. *Bacteroides oralis* Ig4a (Takahashi, 1982) was used as a source of the D-glucanases. The cells were grown anaerobically in either trypticase soy broth (without dextrose, BBL) supplemented with 0.5% dextran (TS-D broth), or Luria broth supplemented with 0.5 mM-isopropyl β -D-thiogalactoside.

Enzyme extract. Subcellular fractions were prepared by the osmotic shock method of Nossal & Heppel (1966). After the osmotic shock step, the cells were suspended in 20 mM-Tris/HCl, pH 7.0, maintained at 0 °C, and were disrupted using a Polytron type PT10/35 sonicator (Kinematica, Luzern, Switzerland) at maximum output for 5 min. After removal of the unbroken cells by centrifugation at 3000 g for 10 min at 4 °C, the supernatant was recentrifuged at 20000 g for 20 min. The pellet was resuspended in 20 mM-Tris/HCl, pH 7.0 and washed twice in the same buffer; it was termed the membrane fraction to indicate membrane and/or membrane associated ribosomes that were not sedimented by the centrifugation at 3000 g. The supernatant was then centrifuged once more at 120000 g for 120 min, to separate the ribosomal and cytoplasmic fractions. The ribosomes were suspended in 20 mM-Tris/HCl, pH 7.0, and washed in the same buffer.

Spheroplasts were formed by treatment with lysozyme (Neu & Heppel, 1964) for 15 min and stabilized by the addition of MgCl₂ to give a concentration of 0.01 M. The spheroplasts were separated from the buffer (20%, w/v,

sucrose, 30 mM-Tris/HCl, pH 7.0) by centrifugation at 13000 *g* for 15 min, and then osmotically lysed in cold distilled water. The suspension was centrifuged at 10000 *g* for 10 min, and the supernatant from the lysed spheroplasts was collected. The pellets were resuspended in 10 mM-Tris/HCl, pH 7.0, and sonicated. Samples of each fraction were dialysed for 24 h against 20 mM-phosphate buffer, pH 7.0, and stored at -20°C .

Substrates. Pseudonigeran, a (1 $\rightarrow$ 3)- α -D-glucan, was extracted from mycelium of *Aspergillus niger* ATCC 16888 as described by Hasegawa *et al.* (1968), and was used for the assay of mutanase. Dextran T-2000, a (1 $\rightarrow$ 6)- α -D-glucan, was purchased from Pharmacia, and was used as a substrate for the dextranase.

Enzyme assays. The D-glucan-hydrolysing activity in each fraction was assayed by measuring the release of reducing groups from the appropriate substrates (Somogyi, 1952). Alternatively the release of D-glucose was measured by using D-glucose oxidase (Dahlqvist, 1961). Unless otherwise noted, the reactions were performed by the addition of 0.5 ml of a suitably diluted enzyme solution to 0.5 ml 20 mM-phosphate buffer, pH 7.0, containing 0.2% dextran, and incubating at 37°C for 30 min (Takahashi, 1982). The mutanase activity was estimated in the same way except that a 0.2% suspension of pseudonigeran was used as the substrate instead of dextran (Hasegawa *et al.*, 1968). One unit of activity (U) was defined as the amount of enzyme which liberated 1 μmol of reducing groups measured as glucose equivalents under standard assay conditions.

Miscellaneous procedures. Under standard assay conditions, protein was estimated by the Lowry method with bovine serum albumin as the standard. The purity of the subcellular fractions (Heppel, 1971) was assessed by assaying for alkaline phosphatase (Malamy & Horecker, 1961), principally a periplasmic enzyme, and β -D-galactosidase (Zipser, 1963), principally a cytoplasmic enzyme. The various D-glucan-hydrolysing enzymes in the cytoplasmic and periplasmic fractions of *B. oralis* Ig4a were isolated by sequential chromatography on DEAE-cellulose and Bio-Gel A-0.5 m (Bio-Rad) as described previously (Takahashi, 1982).

RESULTS

When *B. oralis* Ig4a was grown in TS-D broth after 48 h, most of the dextranase activity (approximately 75%) was recovered in the culture supernatant and the rest remained in the cells (Fig. 1); in contrast, the bulk of the mutanase activity remained bound to the cells. Small amounts of both enzymes were detected in the culture supernatant at the late stationary phase of growth; however, no mutanase activity was detected in the buffer used for washing intact cells.

The intracellular localization of these enzymes was investigated by using two cell fractionation procedures. When *B. oralis* Ig4a cells were converted to spheroplasts by osmotic shock, 60 to 70% of the cell-bound dextranase was released into the buffer within 10 min. Thereafter no more dextranase was released but some (20 to 30%) remained bound to the spheroplasts. This dextranase was of high specific activity and could not be removed from the spheroplasts by washing. Almost all of the mutanase activity (>95%) remained associated with the spheroplasts. Subcellular fractions were obtained by centrifugation, and the various enzyme activities were measured. Most of the dextranase activity was detected in both the periplasmic and cytoplasmic fractions, but not in the membrane or ribosomal fractions (Table 1). In contrast, 70% of the mutanase activity was found in the cytoplasm.

We determined the extent of liberation of the cell-bound D-glucanases during spheroplast formation and spheroplast lysis by using marker enzymes. Alkaline phosphatase was used as a marker enzyme for the periplasmic space, while β -D-galactosidase was used as a marker for the cytoplasm. If a periplasmic enzyme is bound tightly to components of the cell surface outside the cytoplasmic membrane, it could be retained by cells subjected to osmotic shock. Activities released from the cells during spheroplast formation and those released only upon the subsequent lysis of the spheroplasts were assayed. Alkaline phosphatase was largely released during spheroplast formation, whereas β -D-galactosidase was retained (Table 2). Similarly, over 60% of the total dextranase activity was released into the spheroplast-forming buffer, the rest (20–30%) remaining bound to the spheroplast. In contrast, mutanase behaved like a cytoplasmic enzyme and was retained inside the spheroplasts.

The results obtained with the dextranase suggested that there might be two forms of this enzyme, each localized within a different region of the cell. In order to examine this possibility, enzymes in the periplasmic and cytoplasmic fractions were separated by sequential chromatography on DEAE-cellulose and Bio-Gel A-0.5 m columns (Takahashi, 1982). Fractions containing enzyme activity were pooled, concentrated in an Amicon pressure cell with a Diaflo PM-10 membrane, and dialysed against 20 mM-phosphate buffer, pH 7.0. The retentate

Fig. 1. Production of dextranase and mutanase by *B. oralis* Ig4a grown in TS-D broth. Five 1 litre culture bottles containing 800 ml of this medium were incubated anaerobically with 5 ml preincubated bacteria for the time indicated. The total content of one culture bottle was used for the determinations at each time point: dextranase ($\diamond$) and mutanase ($\circ$) in the culture medium and cell-associated dextranase ($\square$) and mutanase ($\triangle$) and cell dry weight ($\blacksquare$) were measured.

Table 1. D-Glucanase localization in fractions prepared by osmotic lysis of *Bacteroides oralis* Ig4a

Subcellular fraction	Enzyme activity [U (mg cell dry wt or equivalent) ⁻¹]		Activity* (%)		Specific activity [U (mg protein) ⁻¹]		Activity of marker enzymes*† (%)	
	Dextran- ase	Mutan- ase	Dextran- ase	Mutan- ase	Dextran- ase	Mutan- ase	Alkaline phos- phatase	β -Galacto- sidase
Stage I shock fluid	5.2	1.2	8	5	0.5	0.01	5.2	1.1
Periplasmic fraction	33.4	1.0	53	4	8.0	0.02	87.3	5.4
Cytoplasmic fraction	23.0	19.6	37	72	7.2	5.7	4.5	81.0
Membrane fraction	0.4	3.8	1	14	0.1	0.8	1.6	1.5
Ribosome fraction	0.7	1.5	1	5	0.3	0.1	1.4	11.0

* Activities are expressed as a percentage of the total activity of the five fractions.

† Overall recovery of alkaline phosphatase was 95.3% and of β -galactosidase, 92.5%.

Table 2. Release of enzymes after spheroplast formation of *Bacteroides oralis* Ig4a

Fraction	Activity of glucanases (%)		Specific activity [U (mg protein) ⁻¹]		Activity of marker enzymes (%)	
	Dextranase	Mutanase	Dextranase	Mutanase	Alkaline phosphatase	β -Galacto- sidase
Supernatant, after removal of spheroplasts	62.8	11.1	16.5	0.8	92.5	15.7
Supernatant, osmotically lysed spheroplasts	28.0	81.8	12.1	6.8	7.1	81.4
Sonic extract of pellet from lysed spheroplasts	9.2	7.1	1.2	0.7	0.4	2.9

was applied to a column of DEAE-cellulose equilibrated with the same buffer, and eluted with 400 ml of a linear salt gradient (0–0.4 M-NaCl). As described previously (Takahashi, 1982), this step resulted in the separation of two dextranases, which were eluted by 0.18 M-NaCl (En-I) and by 0.25 M-NaCl (En-II). A minor peak comprising 10% of the total dextranase activity in the periplasmic fraction was thought to be caused by contamination with cytoplasmic fractions (Fig. 2a, b). The active fractions corresponding to En-I or En-II were pooled and chromatographed on a Bio-Gel A-0.5 m column equilibrated with 20 mM-phosphate buffer, pH 7.0. The dextranases in the periplasmic and cytoplasmic fractions had different molecular weights (Fig. 2c, d).

Fig. 2. (a, b). DEAE-cellulose column chromatography of the periplasmic (a) and cytoplasmic (b) dextran-hydrolysing activities. The cellular fractions were prepared from *B. oralis* Ig4a as described in the text. Both the periplasmic and cytoplasmic fractions were concentrated by ultrafiltration using a PM-10 membrane and dialysed against 20 mM-phosphate buffer (pH 7.0). The retentate was applied to a DEAE-cellulose column. The enzyme was eluted with a linear NaCl gradient (0–0.4 M) in 400 ml of 20 mM-phosphate buffer, pH 7.0, and 3 ml fractions were collected. (c, d) The active fractions eluted from the DEAE-cellulose columns (a) and (b) respectively were pooled, concentrated, and submitted to chromatography on Bio-Gel A-0.5 m columns equilibrated and developed with 20 mM-phosphate buffer (pH 7.0). V_0 , void volume (blue dextran 2000).

DISCUSSION

We have found that the dextranase of *Bacteroides oralis* Ig4a is localized in both the periplasmic and cytoplasmic regions of the cell. About 75% of the dextranase activity of *B. oralis* Ig4a is found extracellularly, but about 60% of the cell-bound enzyme is released when spheroplasts are prepared. In a previous study (Takahashi, 1982), we reported that *B. oralis* Ig4a produces two kinds of dextran-hydrolysing enzyme, designated as En-I (mol. wt 52000) and En-II (mol. wt 44000). The former is an endo-dextranase that produces oligosaccharides, whereas the latter is an exo-enzyme producing glucose as the sole end-product (Takahashi, 1982). The dextranase localized in the periplasmic space corresponded to En-I, and the dextranase localized in the cytoplasm corresponded to En-II.

Walker *et al.* (1981) reported that *Streptococcus mutans* produces two types of dextranase; one was an exo-type of enzyme which was found mainly intracellularly and was almost undetectable in the culture filtrate, whereas the other was an endodextranase which was released in the early exponential phase of growth. Furthermore, Staat & Schachtele (1976) reported that *Bacteroides ochraceus* isolated from dental plaque produced exo- and endo-dextranases with distinct pH optima. However, the localization and physiological function of those two types of dextranase were not described.

The mutanase of *B. oralis* Ig4a was exclusively localized in the cytoplasm, as neither the culture supernatant nor the spheroplast-forming had detectable enzyme activity. Linder *et al.* (1983) reported that the dextranase of *Streptococcus mitis* was localized in the cytoplasm, and was not released into the medium when cells were converted to spheroplasts by the cell wall-degrading M-1 enzyme isolated from *Streptomyces globisporus*. However, Heppel (1971) obtained evidence by use of the osmotic shock technique that many hydrolytic enzymes are localized in the periplasm of Gram-negative bacteria. An accumulated enzyme pool could be

held between the cytoplasmic membrane and an outer membrane (periplasmic space), either bound directly to some component or by being unable to diffuse through the complex outer surface layer (Heppel, 1971; Neu & Heppel, 1965). However, there have been few reports on the localization of polysaccharidases on the surface of Gram-negative bacteria. The mechanism of degradation of exogenous substrates that are unable to pass through the inner membrane has not been clarified. The different subcellular localization of these enzymes may suggest that the polysaccharides in dental plaque are hydrolysed by extracellular enzymes such as En-I to produce oligosaccharides that are small enough to enter the cells. Intracellular polysaccharidases (En-II and mutanase) then further hydrolyse these oligosaccharides to monosaccharides, thus permitting the use of parts of the dental plaque polysaccharide for growth. Therefore, these enzymes may reduce the accumulation of dental plaque and may prove to be useful for the prevention of dental caries in humans.

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