Helicobacter pylori catalase

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Helicobacter pylori is the major aetiological agent of gastrroduodenitis in humans. Due to the potential importance of catalase in the growth and survival of Helicobacter pylori on the surface of inflamed mucosae, we have characterized catalase from H. pylori as a prelude to further studies on the function of the enzyme in vivo. The catalase activity of H. pylori was significantly affected by the presence of blood, serum or erythrocytes in the growth medium: the greatest activity was expressed when the bacterium was grown on medium containing serum. H. pylori catalase is a tetramer with a subunit Mø of 50000. The enzyme had a pH of 9.0–9.3, was active over a broad pH range and was stable at 56°C. It was non-competitively inhibited by sodium azide, and had no detectable peroxidase activity. The Km for the purified catalase was measured as 43 ± 3 mM-H2O2 and the V as 60 ± 3 mmol H2O2 min⁻¹ (mg protein)⁻¹. The native catalase has absorption maxima at 280 nm and 405 nm with further minor shoulders or peaks at 510 nm, 535 nm and 625 nm, consistent with the presence of an iron-porphyrin prosthetic group.

Introduction

Catalase (EC 1.11.1.6) is an important enzyme for the protection of bacteria against the damaging effects of hydrogen peroxide. Although catalase activity does not always correlate with resistance to exogenous H2O2, this enzyme is important to some bacteria in resisting the effects of toxic oxygen species released during inflammatory processes, particularly those involving polymorphonuclear leucocytes (Schwartz et al., 1983; Beaman et al., 1985; Wilson & Weaver, 1985).

Helicobacter (Campylobacter) pylori is the pathogen primarily responsible for gastrroduodenitis in humans (Goodwin et al., 1986; Hazell et al., 1986a, b). In adults H. pylori gastritis begins with an acute inflammatory response (predominantly polymorphonuclear leucocytes) followed by infiltration with mononuclear cells. This results in the characteristic inflammatory lesion associated with H. pylori infection, acute on chronic gastritis (Morris & Nicholson, 1987; Frommer et al., 1988) which persists despite the cellular and humoral response to the presence of the organism (Jones et al., 1984). The mechanisms whereby H. pylori is able to thrive in such an apparently hostile environment remain to be elucidated. Because of the potential role played by catalase in maintaining a stable environment for the growth of H. pylori in vivo, we have characterized the enzyme as a prelude to a better understanding of its role as a putative virulence factor of H. pylori. Part of this work was presented as a poster at the Vth International Campylobacter Workshop, Puerto Vallarta, Mexico, 1989.

Methods

Bacteria. Helicobacter pylori strains were isolated from gastric biopsies obtained from patients undergoing endoscopy at the Veterans’ Affairs Medical Center, Houston, Texas. Strains were characterized as previously described (Hazell et al., 1986b). Campylobacter jejuni strains were obtained from the hospital microbiology department culture collection. H. pylori isolates were designated 8826 (laboratory-adapted isolate), 8801, 8802, 8803 and 8804 (fresh isolates).

Culture conditions. Cultures were incubated at 37°C in an environment of 10% (v/v) CO2 in air and 99% relative humidity. Unless otherwise specified, cultures were grown on 5–7% (v/v) horse blood agar. When testing for the effect of blood products on catalase activity H. pylori was grown on a semi-defined basal medium [Iso-Sensitest Agar (ISA), Oxoid] and ISA supplemented with either 5% citrated human blood (blood agar), 5% human serum (ISA/serum) or 5% human erythrocytes washed in Alsevier’s solution (ISA/RBC) as previously described (Hazell et al., 1989). The blood or blood products were obtained fresh from a healthy H. pylori-negative volunteer.

Catalase extraction and chromatography. H. pylori cells were washed from agar in 0.1 M-potassium phosphate buffer, pH 7.35, containing 0.005% merthiolate (Eli Lilly & Co) (buffer A) and 10 μg ml⁻¹ leupeptin (Boehringer) (buffer A-L). The cells were centrifuged at 17000 g for 8 min, resuspended in buffer A-L and disrupted by three cycles through a French pressure cell. This extract was then centrifuged at 17000 g for 8 min, and the supernatant was recovered and recentrifuged at 75000 g for 45 min. In the case of isolate 8826, due to

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the presence of excess insoluble material that interfered with the chromatography, the final supernatant was mixed 50:50 with Percoll (Pharmacia) and centrifuged at 39000 g for 20 min. Insoluble material was discarded.

The final supernatant or the final supernatant in Percoll was applied to a K 26/100 gel filtration column of Sephacryl S-300 HR (Pharmacia) using buffer A as the liquid phase at a flow rate of 0.5 ml min⁻¹ and eluted. The catalase-positive fractions were pooled and concentrated 10-fold (Amicon Centriprep 30 concentrator, W. R. Grace & Co., Danvers, MA, USA). The concentrate was brought back to the original volume with deionized distilled water. The crude catalase was filtered through the Sephacryl S-300 HR column. Merthiolate was removed by rapid dialysis against 0.1 m-phosphate-buffered saline (PBS), pH 7.35; using the Amicon Centriprep 30 concentrator. The merthiolate-free catalase was filter-sterilized (0.2 μm pore size membrane), and then applied to a Dowex 50W-X8 (now Millipore) MEmSEP 1000-CM ion-exchange capsule (Phenomenex, Rancho Palos Verdes, CA, USA) previously equilibrated with 1/10 concentration buffer A. After washing to remove unbound proteins, the catalase was eluted with a linear gradient generated by 1 M-KCl in 1/10 buffer A. The catalase was then rechromatographed through the Sephacryl S-300 HR column. Merthiolate was removed by rapid dialysis against 0.1 m-phosphate-buffered saline (PBS), pH 7.35, using the Amicon Centriprep 30 concentrator. The merthiolate-free catalase was filter-sterilized (0.2 μm pore size membrane) and stored in the dark at 4 °C.

**Determination of catalase activity.** Qualitative catalase activity was tested by adding 50 μl of the test material to 100 μl of a 3% (v/v) H₂O₂ solution in a microtitre plate and watching for the rapid evolution of oxygen.

Quantitative catalase activity was determined after the method of Beers & Sizer (1952). Solutions of 10–200 μl of 30% (v/v) H₂O₂ in 50 ml of 0.1 m-PBS, pH 7.35 at 25 °C were prepared and the concentration of each solution determined spectrophotometrically using a molar absorption coefficient of 43.481 mol⁻¹ cm⁻¹ at 240 nm. Reaction kinetics of isolated catalase were determined by mixing 100 μl enzyme in 2.9 ml H₂O₂ solutions with the initial reaction rates recorded using a programmable printer (Spectrophotometer 252, Gilford Instrument Laboratories). All assays were repeated to give 12 rate determinations for the first minute of reaction and these were recorded as the rate of decomposition of H₂O₂ min⁻¹ (mg protein)⁻¹. Kₘ and V values were determined by Lineweaver-Burk plots. The line of best fit and regression analysis was computed using Microstat (Ecosoft Inc., Indianapolis, IN, USA).

Comparisons of the catalase activities of whole bacteria were made using 100 μl of a cell suspension consisting of bacteria washed in ice-cold PBS and adjusted to OD₅₀₀ = 1.0. A standardized solution of H₂O₂ in PBS (A₄₆₀ = 0.55) at 25 °C was used for these assays with the reaction rate determined as above. Data from experiments using whole cells were compared using analysis of variance.

**Characterization of catalase.** The Mₑ of the native catalase was determined by gel filtration through fresh Sephacryl S-300 HR in 0.1 m-potassium phosphate buffer, pH 7.35, without merthiolate – which can interact with some proteins in the column – as compared to gel filtration Mₑ markers (Sigma). The subunit Mₛ was determined by SDS-PAGE under fully denatured and reduced conditions using SDS Mₛ markers (Bio-Rad). The isoelectric point was measured on a PhastGel IEF polyacrylamide gel (Pharmacia). Proteins were stained with Coomassie brilliant blue R dissolved in methanol/acetic acid.

 Peroxidase activity was assayed spectrophotometrically using 4-chloro-l-naphthol/H₂O₂, dissolved in 0.05 m-Tris/HCl, pH 7.5, containing 0.2 m-NaCl, as substrate. Purified enzyme (100 μl) was mixed with 100 μl of substrate solution in the well of a microtitre plate and the plate, with the inclusion of appropriate controls, was incubated for 30 min in the dark at room temperature. Colour development was measured on a Titertek Multiskan instrument (Flow Laboratories).

The pH optimum of H. pylori catalase was tested in 0.1 m-potassium phosphate over a pH range of 5.25–8.95. Heat lability of H. pylori catalase in 0.1 m-PBS, pH 7.35, was tested by heating the enzyme in a water bath at 56 °C for 60 min. Aliquots were removed at 10 min intervals and their catalase activity measured quantitatively. The effect of sodium azide on catalase kinetics was tested at a concentration of 50 μm-sodium azide in the presence of 0.25 ng catalase. The reaction rate was determined as outlined above. Reversibility of azide inhibition was tested after dialysis of azide-treated catalase against 0.1 m-PBS, pH 7.35 (three changes of buffer over 18 h at 4 °C). The spectrophotometric profile of the catalase was determined by scanning both the native protein and enzyme treated with K₃Fe(CN)₆/KCN (Tentori & Salvati, 1981) in the range 260–680 nm.

**Protein determination.** Protein estimations were made by either the bicinchoninic acid (BCA) or the microBCA protein assay systems (Pierce Chemical Co.). Bovine serum albumin was the protein standard.

**Results**

**Effect of blood and blood products on catalase expression by H. pylori**

The whole-cell catalase activity of H. pylori isolate 8826 grown on ISA, blood agar, ISA/serum and ISA/RBC over 96 h determined that the length of incubation and the type of blood product in the medium had a significant effect (P < 0.001) on the catalase activity (Fig. 1). This

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Fig. 1. Effect of incubation period and medium supplements on (a) the yield of cells and (b) the expression of catalase by whole cells of H. pylori strain 8826 when grown on basal medium (ISA), (C) and basal medium containing human serum (ISA/serum), (C), blood (blood agar) (●) or erythrocytes (ISA/RBC) (■). Catalase activity is also shown for two strains of C. jejuni, 1186 (△) and 1284 (▲), grown on blood agar. The results in (b) are plotted as means ± 95% confidence intervals.
medium effect was also apparent with four other isolates (8801, 8802, 8803 and 8804) as determined using whole cells following 3 d incubation (Fig. 2). The whole-cell catalase activity of two strains of *C. jejuni* tested concurrently was about five times less active than *H. pylori* (*P* < 0.001) at the same specified H2O2 concentration (Fig. 1).

Characterization of catalase

*H. pylori* catalase was purified from two isolates (8826 and 8801) to the order of 65-fold. The catalase from both isolates had identical properties; representative data from isolate 8826 are given below. The native catalase had an apparent *M*, of 190 000–200 000 as determined by gel filtration, whereas the reduced denatured enzyme had an apparent *M*, of the order of 50 000. The isoelectric point was in the range 9.0–9.3. The *H. pylori* catalase was active over a broad pH range, showing no significant difference in activity between pH 6.5 and 8.95. It was stable at 56 °C for 1 h and was reversibly inhibited by sodium azide. Kinetic data were consistent with this inhibition being non-competitive. No peroxidase activity was detected.

The kinetic properties of the catalase from *H. pylori* were determined; the *K*ₘ was measured as 43 ± 3 mm- H₂O₂ and the *V* as 60 ± 3 mmol H₂O₂ decomposed min⁻¹ (mg protein)⁻¹.

The native catalase has absorption maxima at 280 nm and 405 nm (Soret band) and further minor shoulders or peaks at 510 nm, 535 nm and 625 nm, and a 405/280 ratio of 0.65. Treatment with K₃Fe(CN)₆/KCN produced a spectral shift of the 405 nm peak to 422 nm (Fig. 3).

Discussion

Catalase is a ubiquitous enzyme found in eukaryotic and most prokaryotic organisms. The native *H. pylori* catalase appears to be a tetramer (*M*, 200 000) and the spectral analysis reported here is consistent with the presence of an iron–porphyrin prosthetic group (Hochman & Shemesh, 1987). The enzyme is soluble and may be distributed throughout the cytosol and in the periplasmic space (unpublished results). Whether the catalase is loosely associated with specific membrane proteins remains to be determined.

*H. pylori* catalase was reversibly and non-competitively inhibited by sodium azide. Azide combines reversibly with iron–porphyrin prosthetic groups and causes a reduction of the iron to the ferrous state (Keilin & Hartree, 1945; Lamberg & Foulkes, 1948). Azide and cyanide may appear as non-competitive catalase inhibitors; however, under conditions of high substrate concentration they may act as competitive inhibitors (Ogura, 1955). The enzyme was heat stable and had a broad pH activity similar to that seen with *Klebsiella pneumoniae* (KpT) and *Escherichia coli* (HPII), the latter having two distinct activity peaks at pH 6.8 and 10.5 (Meir & Yagil, 1985; Goldberg & Hochman, 1989). Unlike the *E. coli* catalases (HPII and HPI; Meir & Yagil, 1985), no concomitant peroxidase activity was detected for *H. pylori* catalase. Heat stability is a property shared by the non-peroxidase catalases (i.e. the 'typical' catalases) and the characteristics of *H. pylori* catalase are consistent with the enzyme being a ‘typical’ catalase.

The isoelectric point of the *H. pylori* catalase (9.0–9.3) appears unique. The isoelectric point of catalase usually occurs at an acid pH (e.g. *Rhodopseudomonas capsulata*,...
4.5, and Pseudomonas pyrocina, 5.0; Weisner et al., 1985; Hochman & Shemesh, 1987). This characteristic of the enzyme may represent an adaptation to a unique environment.

In media supplemented with different blood products, there was a fall in the catalase activity of H. pylori as the relative concentration of erythrocytes increased and that of serum decreased. This is consistent with either a serum factor(s) stimulating catalase activity and/or the presence of haem-containing proteins, released from erythrocytes by the action of H. pylori (Wetherall & Johnson, 1989), suppressing the synthesis of catalase. Suppression of synthesis of porphyrins by haem groups has been demonstrated in mature mammalian cells; and in rodents, liver catalase activity increases subsequent to serum injection (Weil-Malherbe & Schade, 1948; Woods, 1974). In E. coli, the expression of catalase activity is subject to catabolite repression in the presence of glucose (Meir & Yagil, 1990). Whether glucose, or other compounds, can repress the catalase activity of H. pylori, which is non-glycolytic, remains to be determined. Also, unlike E. coli (Meir & Yagil, 1990), the expression of catalase in H. pylori was not enhanced by the cells entering stationary phase, as the highest activity occurred in young cultures. Two or possibly three catalases have been identified in E. coli (Meir & Yagil, 1985; Peyru & Fraenkel, 1968). In the studies reported here we have identified one isomer of catalase. Whether other isomers of catalase can be induced in H. pylori under conditions such as oxidative stress remains to be determined.

The catalase activity of H. pylori was significantly greater than that of the related bacterium C. jejuni. H. pylori is a major aetiological agent of human acute on chronic gastritis and a pronounced catalase activity may aid the survival of the bacterium in vivo. Beaman et al. (1985) noted that resistance of Nocardia asteroides to the microbicidal activity of polymorphonuclear leucocytes, which can release H2O2 both intra- and extracellularly (Clifford & Repine, 1982), was mediated by both superoxide dismutase and catalase. However, the presence or absence of catalase does not render absolutely a bacterium more or less susceptible to the lethal effects of H2O2, as susceptibility to H2O2 can also be dependent upon the sensitivity of cellular components to oxidative damage (Schwartz et al., 1983; Wilson & Weaver, 1985).

We (Hazell et al., 1989; Hazell & Graham, 1990) have previously demonstrated that bovine liver catalase can aid the growth of H. pylori in vitro, this being due, in part, to the prevention of formation of toxic peroxidized fatty acids. These data would indicate that H. pylori is at least indirectly sensitive to oxidative damage. Whereas endogenous catalase of H. pylori does not appear to play a role in conditioning the external environment, it may be critical at the cellular level.

The K_m for H. pylori catalase was 3–10 times higher than that recorded for most other bacteria, but about one-third that of heat-stable mycobacterial catalase. Also, the enzyme was stable at concentrations of H2O2 known to inhibit other bacterial catalases, a property shared with several species of the genus Mycobacteria (Wayne & Diaz, 1982; Meir & Yagil, 1985; Allgood & Perry, 1986; Hochman & Shemesh, 1987; Goldberg & Hochman, 1989). These properties could relate to the adaptation of the mycobacterial and H. pylori enzymes to an environment rich in toxic oxygen species.

This paper provides fundamental information on the nature and activity of H. pylori catalase and is a prelude to further studies that will determine if this enzyme is important in enabling H. pylori to survive on the surface of inflamed gastroduodenal mucosae.

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