Borrelia burgdorferi possesses a collagenolytic activity

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Abstract

Lyme disease is a multisystemic disorder caused by Borrelia burgdorferi, an invasive spirochete. B. burgdorferi has a predilection for collagenous tissue and one major clinical manifestation of the disease is arthritis. We have identified a collagenolytic activity in B. burgdorferi detergent lysates using iodinated gelatin as well as iodinated pepsinized human collagen types II and IV as protein substrates. In addition, we describe several proteolytic activities in B. burgdorferi with molecular masses greater than 200 kDa on sodium dodecyl sulfate polyacrylamide gels containing copolymerized gelatin. We propose that the collagenolytic activity of B. burgdorferi has a role in invasion, in the pathogenesis of Lyme arthritis, and perhaps also in other manifestations of Lyme borreliosis.

Keywords: Borrelia burgdorferi; Lyme disease; Collagenolysis

1. Introduction

Borrelia burgdorferi, the spirochete that causes Lyme disease, is an invasive bacterium that is transmitted to humans and other animals by the bite of infected Ixodes scapularis ticks. Following infection, spirochetes remain in the periphery of the site of the tick bite for a limited time, after which they disperse to multiple organs, including the nervous, musculo-skeletal, reticulo-endothelial and cardiovascular systems, as well as the eye [1].

The molecular mechanism for tissue invasion by B. burgdorferi has remained a mystery for some time. It had been assumed that B. burgdorferi does not produce any hydrolytic activities needed to degrade the extracellular matrix. Recently, there have been several exciting reports describing the ability of B. burgdorferi to bind to plasminogen, the zymogen of the serine protease plasmin [2–5]. The bound plasminogen could be activated to plasmin by host-derived plasminogen activators [2–5]. Putative plasminogen receptors include a 70 kDa protein [5] as well as OspA [2,3,5].

There is little doubt that plasmin(ogen) plays a role in invasion of tissues by B. burgdorferi. However, it is especially pertinent that B. burgdorferi has a predilection for collagenous connective tissue [6]. Spirochetes have been detected between collagen fibers of bradytrophic dense connective tissue [7] as well as other collagen-rich sites [8]. This manifest predilection for collagenous tissues prompted us to search for a collagenase-like activity in extracts of
2. Materials and methods

2.1. Materials

Young rabbit serum was obtained from Pel Freeze Biologicals (Rogers, Arkansas). Sephadex G-25 M PD-10 columns were obtained from Pharmacia LKB Biotechnology AB (Uppsala, Sweden). Gelatin for zymography was obtained either from Difco laboratories (Detroit, MI) or J.T. Baker Chemical Co. (Phillipsburg, NJ). All other reagents used for sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) were from Bio-Rad Laboratories (Hercules, CA). Protease-free gelatin, pepsinized human collagen types I, II, III, and IV, gelatin-free BSK-H medium, trypsin-TPCK (trypsin treated with \(\alpha\)-1-tosylamide-2-phenylethyl chloromethyl ketone), TLCK (N-\(\alpha\)-p-tosyl-L-lysine chloromethyl ketone), EDTA (ethylenediaminetetraacetic acid) and all other biochemicals were from Sigma Chemical Company (St. Louis, MO).

2.2. In vitro cultivation of B. burgdorferi

B. burgdorferi isolates were routinely cultured at 34°C (in 5% CO\(_2\), 3% O\(_2\) and 92% N\(_2\)) in gelatin-free BSK-H medium [9] supplemented with 10% heat-inactivated young rabbit serum. Low-passage isolates (below 12) of several strains were used.

2.3. Preparation of \(^{125}\text{I}-\)labeled gelatin or human collagen types II and IV

Pepsinized human collagen types II and IV were solubilized in 20 mM acetic acid, 100 mM NaCl, pH 3.3. The proteins were further purified by HPLC-gel permeation chromatography on a 10 × 300 mm Bio-Sep 2000 (Phenomenex, Torrance, CA) column equilibrated in 2% glycerol in phosphate buffered saline (PBS), pH 7.4. The flow rate was 2 ml/min. The proteins were iodinated using the Iodo-Beads iodination reagent according to the manufacturer’s instructions (Pierce, Rockford, IL). Briefly, the proteins (160–200 \(\mu\)g in 1 ml PBS) were incubated at ambient temperature in the presence of 0.6 mCi Na\(^{25}\text{I}\) and Iodo-Beads (one bead per reaction). After 15 min, the protein samples were passed through a Sephadex G-25 M PD-10 column to remove free iodine and other small reaction products. The specific activities for \(^{125}\text{I}\)-labeled gelatin, collagen type II and collagen type IV were 812 000, 215 000 and 354 000 cpm/\(\mu\)g protein respectively.

2.4. Analysis of collagenolytic activity

To assess proteolytic activity with iodinated gelatin or collagen substrates, B. burgdorferi was solubilized in PBS containing 1–5% (w/v) CHAPS (3-[\(\alpha\)-cholamidopropyl]dimethylammonio]-1-propanesulfonate), a zwitterionic detergent. Typically, extracts of solubilized B. burgdorferi of 0.3–2.5 × 10⁸ spirochetes in 100 \(\mu\)l CHAPS buffer were incubated with 1–5 \(\mu\)g iodinated protein in a final volume of 100 \(\mu\)l for 18 h at 37°C. Assay blanks not containing enzyme were also done. Sodium azide (0.05–0.25%) was added to prevent bacterial contamination during the long incubations. The reaction (in duplicate) was stopped by the addition of 1/2 volume 0.04 N phosphotungstic acid in 2 N HCl [10]. After 15 min at ambient temperature the samples were centrifuged at 17 000 \(\times g\) for 15 min in an MR1812 Jouan microfuge to precipitate any undegraded gelatin/collagen and the acid soluble phase counted for released radioactivity. The activity is expressed as the mean acid soluble cpm released in the presence of spirochete protein minus the blank controls.

Analysis of collagenolytic activity by zymography was performed in gelatin-containing SDS-PAGE as described by Lacks and Springhorn [11]. The B. burgdorferi proteins were solubilized in sample buffer [12] containing 2% SDS and 2.3 M urea without \(\beta\)-mercaptoethanol (2-ME), but were not boiled, to avoid irreversible denaturation of the putative proteases. SDS-PAGE was done in 16 × 20 cm gels using the Bio-Rad Protein II xi system (Bio-Rad). After electrophoresis, the gel was washed twice (10 min) in H\(_2\)O and incubated overnight in 100 mM HEPES buffer, pH 7.5, to allow for protein renaturation while the SDS diffused out of the gel matrix. The gel was then stained with Coomassie brilliant blue, and destained in 7% acetic acid–30% methanol. Proteolytic activity was revealed by clear areas (bands).
25 μl Bb + Collagen type IV
25 μl Bb + Collagen type II
25 μl Bb + Gelatin
5 μl Bb + Gelatin

Cpm released [x 10^5]

Fig. 1. Hydrolysis of iodinated gelatin and human soluble collagen types II and IV by *B. burgdorferi* detergent lysates. Protease-free gelatin and HPLC-purified human soluble collagens types II and IV (from Sigma) were iodinated using Iodo-Beads. Approximately 2.5×10^9 *B. burgdorferi* JD1 were solubilized in 2.5 ml 10% (v/v) glycerol in PBS containing 5% (w/v) CHAPS and 0.25% sodium azide. After 10 min on ice the sample was centrifuged at 17 400×g for 20 min (4°C). The CHAPS-soluble fractions were assayed for collagenase activity as described in Section 2. 5 μg 125I-labeled gelatin (protease free), soluble human collagens type II, or human collagen type IV were incubated with either 5 or 25 μl CHAPS soluble spirochete fraction representing 5×10^7 or 2.5×10^8 spirochetes/ml respectively. The cpm initially incubated were 2.4×10^6, 8.9×10^5, and 1.6×10^6 cpm (5 μg) gelatin, collagen type II and collagen type IV respectively.

3. Results

We found that *B. burgdorferi* collagenase is active and can be solubilized with detergents such as CHAPS. In addition, the hydrolysis of gelatin or collagens was found to be dependent on both *B. burgdorferi* protein and substrate concentration. 25 μl of a CHAPS soluble *B. burgdorferi* (strain JD1) fraction (from 2.5×10^8 spirochetes) was able to catalyze the hydrolysis of iodinated gelatin, collagen type II and collagen type IV, cleaving approximately 59.8% (1.20×10^6 cpm), 22% (1.96×10^5 cpm) and 11% (1.76×10^5 cpm) respectively, of the added radioactivity (Fig. 1). In the presence of 5 μl CHAPS soluble *B. burgdorferi* (from 0.5×10^8 spirochetes) fraction the release of radioactivity from gelatin was 19.1% (4.6×10^5 cpm) of the added radioactivity (Fig. 1). The amount of hydrolysis is also dependent on gelatin or collagen (type II) concentration (Fig. 2). Using whole (CHAPS solubilized) *B. burgdorferi*, the total amount of radioactivity released from gelatin and collagen type II increased 4.6- and 7.5-fold respectively, when the concentration of radiolabeled protein substrate was increased from 1 μg to 5 μg (Fig. 2). In addition, in the presence of 5 μg [125I]gelatin, increasing the concentration of spirochetes from 0.3×10^8 (total) to 2.5×10^8 (from a CHAPS lysate) also resulted in a 10-fold increase in the release of acid soluble 125I cpm (compare Bb+Gelatin (5 μg) sample in Fig. 2 with 25 μl Bb+Gelatin sample in Fig. 1). Proteolytic activity does not appear to be inhibited by EDTA, but was abolished by heating the *B. burgdorferi* preparation at 95°C (10 min) before addition into the assay (Fig. 2).

The collagenolytic activity of *B. burgdorferi* also
Fig. 2. The effect of gelatin or collagen concentration on *B. burgdorferi* collagenase activity. Whole *B. burgdorferi* JD1 (3x10⁷) was incubated with increasing concentrations of [³⁵S]gelatin or [¹⁴C]human collagen type II (1 µg and 5 µg) in PBS, pH 7.5 containing 0.5% CHAPS and 0.05% sodium azide. Spirochetes heated at 95°C for 10 min prior to the start of the assay were used as controls.

was analyzed by SDS-PAGE zymography. On 7% (7% T/0.6% C) SDS-PAGE gels containing gelatin, a very high molecular mass single band of proteolytic activity for *B. burgdorferi* strain B31 was detected (Fig. 3). However, by reducing the concentration of acrylamide to 6% and by increasing the bis-acrylamide concentration (6% T/1.1% C) it was possible to resolve at least three bands of proteolytic activity in *B. burgdorferi* strains B31, JD1 and 25015 (Fig. 4).

The molecular mass of the enzyme(s) appeared to be greater than the mass of myosin (203 kDa); the enzymatic activity was present in extracts of cells harvested in both stationary and exponential phase growth (Fig. 4). The same three proteolytic activities seen on gelatin gels for *B. burgdorferi* (JD1) were also able to hydrolyze soluble human collagen types I, II, and IV when these were incorporated into the gels (not shown). The three bands were present even when the spirochetes were solubilized in 2% SDS alone or in 2% SDS containing 6.9 M urea prior to SDS-PAGE zymography. No bands of activity were seen when conditioned medium was tested. The enzyme was active at pH 7.5 and, in one experiment, was still active at pH 5. The enzyme(s) was (were) not easily removed from the spirochetes since it was present even after extensive washing in PBS. In addition, the activity of the enzyme does not appear to be significantly affected by EDTA, 2-ME, ATP or divalent cations when these were added into the renaturation buffer in this system (not shown).

To activate any collagenase zymogens we preincubated *B. burgdorferi* with plasmin for 30 min at 37°C prior to the addition of sample buffer and SDS-PAGE. Plasmin did not appear to activate any additional collagenase activity nor did it produce any additional bands. Plasmin itself had no activity. Similar results were found using [³⁵S]gelatin (not shown). In contrast to plasmin, under these experimental conditions (zymography) trypsin-TPCK totally destroyed the *B. burgdorferi* gelatinase (not shown). When *B. burgdorferi* was preincubated with both trypsin and the trypsin inhibitor TLCK
Fig. 4. Gelatinase activity in *B. burgdorferi* B31, JD1 and 20515 in exponential and stationary growth phase. *B. burgdorferi* B31, JD1 and 20515 were run on an SDS-PAGE gel (6% T/1.1% C) containing 680 μg gelatin/ml. 191 μg *B. burgdorferi* protein was loaded per lane (2-7). Lane 1: myosin (203 kDa); lane 2: stationary growth phase *B. burgdorferi* B31; lane 3: exponential growth phase *B. burgdorferi* B31; lane 4: stationary growth phase *B. burgdorferi* JD1; lane 5: exponential growth phase *B. burgdorferi* JD1; lane 6: stationary growth phase *B. burgdorferi* 20515; lane 7: exponential growth phase *B. burgdorferi* 20515.

prior to zymography, full collagenase activity was found. This suggests that the collagenase activity is both trypsin sensitive and is not inhibited by TLCK.

4. Discussion

We have shown that *B. burgdorferi* possesses a collagenolytic activity which can hydrolyze gelatin and several different collagen types. Most importantly, this enzymatic activity is a specific collagenase/gelatinase activity since the telopeptide ends of the collagen substrates had been removed by pepsinization, thereby eliminating detection of non-specific proteases [13]. The collagenase(s) of *B. burgdorferi* that we have identified is unusual because of its size. Most bacterial collagenases have molecular masses less than 120 kDa [10,11,14,15]. The collagenolytic/gelatinolytic activity of *B. burgdorferi* is of high molecular mass (greater than 200 kDa). The enzyme is unlike the collagenase of *Treponema denticola*, which prefers small peptides [16]. In addition, the collagenase is active in the presence of plasmin, indicating that both proteolytic activities can functionally co-exist within the spirochete.

As far as we can ascertain from the literature, this is the first example of a collagenolytic activity described in *B. burgdorferi*. Recent findings by Moser et al. [17] have provided evidence that together, plasminogen/plasmin (after tissue-type plasminogen activation) and metalloproteinases (gelatinase/collagen-
ase) provide the biochemical mechanism for tumor invasiveness by ovarian epithelial carcinoma. Young et al. [18] found that plasma membrane fractions from ovarian adenocarcinoma cells not only promote zymogen activation of MMP-2 (matrix metalloproteinase-2) but also increase the efficiency of gelatinolysis. They hypothesized that even cells that do not express endogenous MMP-2 may be able to utilize exogenous MMP-2 to mediate proteolysis associated with invasion and metastasis. It is also possible that there exist tissue inhibitors of B. burgdorferi collagenase in a manner analogous to the inhibition of progelatinase A and B by tissue inhibitors of metalloproteinases-2 (TIMP-2) and TIMP-1, respectively [10,19]. How B. burgdorferi targets to specific tissues may depend, in part, on whether the tissue can activate or inhibit the bacterial collagenase.

Based on the above findings, and findings in other systems, our working hypothesis is that the B. burgdorferi gelatinase/collagenase(s) activity, together with plasmin(ogen) plays an important role in the process of bacterial dissemination through the skin and endothelium, and probably plays a major role in Lyme arthritis. We hypothesize that B. burgdorferi collagenase(s), when released into the extracellular matrix of cartilage has the potential to dissociate collagen type II fibers. Release of collagen fragments could elicit an autoimmune type of arthritis. It is also possible that B. burgdorferi collagenase may act to degrade not only collagens but the monomeric form of high density cartilage proteoglycan (aggrecan), as has recently been shown by Singer et al. [20] for matrix metalloproteinases, MMP (gelatinase A or stromelysin). Generation of a cleavage product between Asn-341 and Phe-342 (VDIPEN) demonstrated for the first time the MMP-dependent cat
dolysis of aggrecan at sites of chondrodestruction in inflammatory arthritis. In an exciting preliminary finding, it has been shown that B. burgdorferi is able to degrade proteoglycans from human articular cartilage (H.D. Adkisson (Washington University, St. Louis, MO) and D.J. Grab, unpublished results). Whether the B. burgdorferi proteoglycanase and collagenase activities are the same remains to be tested.

In conclusion, many mechanisms have been reported in the scientific literature to explain the ability of B. burgdorferi to bind and penetrate tissues and cells. Proteases such as plasmin [2–5] and collagenase (this paper) may both play a role.

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References


