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Purification and characterization of a B*acillus cereus* collagenolytic/proteolytic enzyme and its effect on *Meloidogyne javanica* cuticular proteins

Sigal Sela[1], Hedva Schickler[2], Ilan Chet[2] and Yitzhak Spiegel[1,*]
[1] *Department of Nematology, ARO, The Volcani Center, Bet Dagan 50250, Israel (Fax: (972-3) 9604180);* [2] *Otto Warburg Center for Agricultural Biotechnology, The Hebrew University of Jerusalem, Faculty of Agriculture, Rehovot 76100, Israel;* * *Author for correspondence*

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Abstract

A novel collagenolytic/proteolytic enzyme isolated from the bacterium *Bacillus cereus* was purified and characterized. The extracellular enzyme was secreted into the growth medium only after induction by collagen. It was purified by two-step chromatography, consisting of gel filtration through a Sephadex G-100 column and then through an anion-exchange column. Molecular mass, as determined by SDS-PAGE was 42.8. The 42.8-kDa collagenase band was eluted from the gel to obtain the purified enzyme. The enzyme was found to have a very wide range of optimal pHs for activity (5.4 – 8.2), and was stable at temperatures between 4 and 40 °C. In addition to its collagenolytic property, the enzyme revealed very strong proteolytic activity, demonstrated by its ability to digest bovine serum albumin. The enzyme's ability to damage nematode cuticles was demonstrated by the digestion of collagens extracted from intact cuticles of second-stage juveniles of the root-knot nematode *Meloidogyne javanica*.

Introduction

Collagenases are a group of Ca^{2+}- and Zn^{2+}-dependent enzymes that can hydrolyze collagens in their natural form (triple helix) at room temperature and physiological pH (Dean and Domnas, 1983; Harper, 1980). Collagenases of various origins are specific to different collagen types (Liotta et al., 1979). Most collagenases, however, also exhibit proteolytic activity and only those isolated from *Clostridium* are considered 'real' specific collagenases (Mandl, 1961). In prokaryotic cells such as *Achromobacter iophagus* (Keil-Dlouba et al., 1976) and *Vibrio* B-30 (Merkel and Dreisbach, 1978), collagenase synthesis is often induced by the presence of native, denatured or high-molecular-weight fractions of collagen in the growth medium. The optimal activity of most collagenases isolated to date has been found within a pH range of 7 to 7.5, although Kawahara et al. (1993) reported a collagenase isolated from *Bacillus alvei* DC-1 with an optimal activity at acidic pH.

Cuticles of the bacteria-feeding nematodes *Caenorhabditis elegans* (Cox et al., 1981) and *Panagrellus silusiae* (Leushner et al., 1979), and animal-parasitic *Ascaris* species (Winkfein et al., 1985), have been extensively characterized. The major structural components of these cuticles are collagens (Kingston, 1991). The entire surface of plant-parasitic nematodes is covered by a multilayered cuticle. Cuticle also lines the mouth, buccal capsule and oesophagus, as well as the rectum and anus (Bird and Bird, 1991). Hence cuticle degradation could be an effective way of controlling pre-parasitic and parasitic forms of root-knot nematodes. The role of collagenolytic activity on plant-parasitic nematodes in the soil was evaluated by applying collagen to the soil. This led to the enrichment of collagenolytic microorganisms which drastically reduced the number of galls caused by *Meloidogyne javanica* on tomato roots (Galper et al., 1989). When added directly to the soil, collagenases and proteases reduced the motility of a *Tylenchorhynchus dubius* population up to 75%; they were

also effective against *Pratylenchus penetrans* (Miller and Sands, 1977). Moreover, root galling caused by *M. javanica* was further decreased when the collagen-amended soil was supplemented with a new isolate of the collagenolytic and elastolytic fungus *Cunninghamella elegans* (Galper et al., 1991). Culture filtrate of this fungus immobilized *M. javanica* second-stage juveniles (J2), inhibited egg hatch and substantially reduced the motility of *Rotylenchulus reniformis* and *Xiphinema index* (Galper et al., 1991). This report describes the purification and identification of a collagenase/protease isolated from *Bacillus cereus*; this bacterium was found to be one of the most dominant microorganisms isolated from soil enriched with collagen (Spiegel, Sharon, Chet and Schickler, unpublished results). This paper also evaluates the enzyme's effectiveness at degrading cuticle collagens of the root-knot nematode *M. javanica*.

Materials and methods

Nematodes

M. javanica J2 were obtained from 2- to 3-month-old tomato plants (*Lycopersicon esculentum* cv. Niagara) which had been inoculated with *M. javanica* J2 3 weeks after sowing (Hussey and Barker, 1973). Eggs were separated from egg masses by incubating in sodium hypochlorite (0.5% v/v, 1 min), and hatched in twofold-diluted PBS (0.5 M phosphate buffer, pH 7.2) to obtain ineffective J2 (Hussey and Barker, 1973).

Bacteria

Bacillus cereus bacteria were previously isolated and identified in Bet Dagan by Oka et al. (1993). Bacterial colonies were grown on nutrient agar. One colony was transferred to 50-ml flasks containing nutrient broth (NB), and grown aerobically in flasks, on a rotating shaker (200 rpm), overnight at 32 °C. This starting material was then transferred to 1 litre flasks containing NB or collagen medium and grown aerobically on a rotating shaker (200 rpm), overnight at 32 °C. The collagen medium contained, per litre, 2 g collagen type I from bovine Achilles tendon (Sigma Chemical Co., St. Louis, MO), 0.1 g $CaCl_2.2H_20$, 0.8 g K_2HPO_4, 0.3 g $mgSO_4.7H_2O$, and 0.1 g yeast extract (Difco, Detroit, MI). To compare growth rate and collagenase production, 1 ml of the starting material was centrifuged, and the growth rate was estimated by weighing the dry pellet.

Enzyme purification

Bacteria were separated from the media (3 litres) by centrifugation (6000xg for 15 min at 4 °C). The supernatant was lyophilized and then redissolved in 80 ml of 50 mM Tris-HCl buffer (pH 7.4, with 5 mM $CaCl_2$), filtered through a cellulose triacetate filter (0.2 mm) and dialyzed overnight against distilled water (2 × 5 litre) at 4 °C. The redissolved, dialyzed crude extract was applied to a 500-ml gel-filtration Sephadex G-100 column (Pharmacia, Uppsala, Sweden). Fractions (5 ml) were eluted at 4 °C using the Tris-HCl-$CaCl_2$ buffer at a flow rate of 50 ml/h, collected and assayed for collagenase activity and protein content.

Collagenase activity was quantitatively determined according to Wunsch and Hendrich (1963): the reaction mixture included 0.5 ml of the *B. cereus* culture filtrate, 2 ml of the synthetic peptide 4-phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-D-Arg (Sigma) which served as an enzyme substrate (2 mg peptide dissolved in 0.02 ml methanol and 1.98 ml Veronal buffer), and 0.25 ml of 50 mM N-ethylmaleimide (NEM), which is a non-specific protease inhibitor. One unit of enzyme activity was determined by comparison with the O.D. obtained using one unit of a commercial collagenase from *Clostridium histolyticum* (Sigma).

Throughout their purification, proteins were monitored by UV detection, i.e. by following their absorbance at 220 and 280 nm. A further quantitative determination was performed using Bradford's (1976) method.

Enzyme characterization: gel electrophoresis

Analytical polyacrylamide gel electrophoresis (PAGE) with 10% gels containing 0.1% (w/v) sodium dodecyl sulphate (SDS) was performed in a Mini-Protein II apparatus (Bio Rad Laboratories, Richmond, CA), using 1.5-mm-thick vertical slab gels, essentially as described by Laemmli (1970). Proteins (5 mg) were dissolved in 25% (v/v) sample buffer, 0.5 M Tris-HCl, pH 6.8, 4% SDS, 0.005% (w/v) Bromophenol Blue, and 20% (v/v) glycerol, boiled for 1 min and loaded onto the gel. Gels were stained overnight with Coomassie Brilliant Blue G-250, washed with 25% (v/v) methanol and then destained for 3 h in a 7% (v/v) acetic acid solution. Low-range, prestained molecular-

weight markers (Bio Rad) were run on each gel, consisting of: phosphorylase b (143 kDa), BSA (97 kDa), ovalbumin (50 kDa), carbonic anhydrase (35 kDa), soybean trypsin inhibitor (30 kDa) and lysozyme (22 kDa).

Molecular-mass determination

The molecular mass of the purified enzyme was estimated by comparison of the SDS-PAGE band in question with the aforementioned low-molecular-weight standards.

Zymography

Enzyme activity was detected by gelatine zymography (Tyagi, 1993). Protein samples (0.5 mg) were mixed with β-mercaptoethanol-free sample buffer and applied to a 10% SDS-polyacrylamide gel which had been co-polymerized with 0.25% (w/v) gelatine (Sigma). The gel was washed with 2.5% (v/v) Triton X-100 at room temperature (RT) to remove the SDS, and incubated overnight at RT in a developing buffer (50 mM Tris, 0.2 M NaCl, 5 mM $CaCl_2$, 0.02% w/v Brij 35, adjusted to pH 7.6 with HCl). Gels were stained overnight with Coomassie Brilliant Blue R-250, and the clear zones appearing on the gel indicated the location of proteins exhibiting proteolytic and/or collagenolytic activities.

Effect of pH and temperature on enzyme activity

The effect of pH was determined using various buffers containing 5 mM $CaCl_2$ and: I. 50 mM citrate buffer, pH 3.1 to 5.2; II. 50 mM Tris-maleate buffer, pH 5.4 to 8.2; III. 50 mM glycine-NaOH buffer, pH 8.6 to 10.2. The effect of temperature was examined by varying it from 4 to 60 °C. In both studies, collagenase activity was assayed with 40 units of the enzyme using the synthetic peptide substrate. One unit of enzyme activity was defined as the amount of enzyme increasing the O.D. value by 1.

Evaluation of nematicidal activity

I. Isolation of collagens from Meloidogyne javanica *cuticle*

Collagens were isolated from cuticles of *M. javanica* J2 essentially as described by Reddigari et al. (1986) with several modifications: 1 ml of nematode suspension was sonicated (5-sec pulses for 3 min) to obtain intact cuticle fragments. These fragments were pelleted and resuspended in two volumes of SDS Tris-Pefabloc buffer, pH 6.8, consisting of 17 mM SDS, 50 mM Tris-HCl, pH 7.4 plus 5 mM $CaCl_2.2H_2O$, and 1 mM Pefabloc (a protease inhibitor). The Tris-Pefabloc buffer contained 10% (w/v) β-mercaptoethanol. The fragments were then boiled in a water-bath at 100 °C for 15 min and held overnight at RT before re-pelleting. The β-mercaptoethanol-soluble proteins in the supernatant were precipitated with nine volumes of cold acetone, stored at $-$ 20°C overnight and pelleted. The pellet, made up of β-mercaptoethanol-free collagens, was resuspended in Tris-HCl buffer and stored at $-$ 20°C.

II. Digestion of M. javanica *cuticle by collagenases*

β-mercaptoethanol-soluble proteins (1.1 mg/ml) were incubated with commercial collagenase from *Clostridium histolyticum* (0.77 U/mg) or with purified *B. cereus* enzyme (0.75 U/mg). The reaction mixture was incubated for 10 min, 2 h or 18 h at 37 °C. As a control, the collagenases were incubated under identical conditions with 0.1 mg/ml bovine serum albumin (BSA) (negative control) or 1.5 mg/ml commercial collagen (positive control) from rat tail or kangaroo (Sigma). To end the reaction, the mixture was added to a sample buffer, heated for 1 min at 100 °C, separated by 10% SDS-PAGE and stained with Coomassie Brilliant Blue G-250.

Results

Isolation and purification of the enzyme

Collagenase production and secretion was most marked in the collagen-based vs. NB medium. The collagenase activity measured in the minimal collagen medium was 29.0 U/ml, whereas in the rich NB medium it was 5.0 U/ml. This, despite the fact that the number of bacteria growing in the latter was twice that in the collagen medium. Collagenase activity was observed in a medium containing NEM, a general inhibitor of proteases other than collagenases. Moreover, the strong induction of the enzyme by collagen medium strengthened the assumption of its collagenolytic activity. The overall amount of protein secreted into the culture filtrate was higher when *B. cereus* was grown in NB as compared to the collagen medium (66.8 and 26.9 mg/litre, respectively). Accordingly, the specific

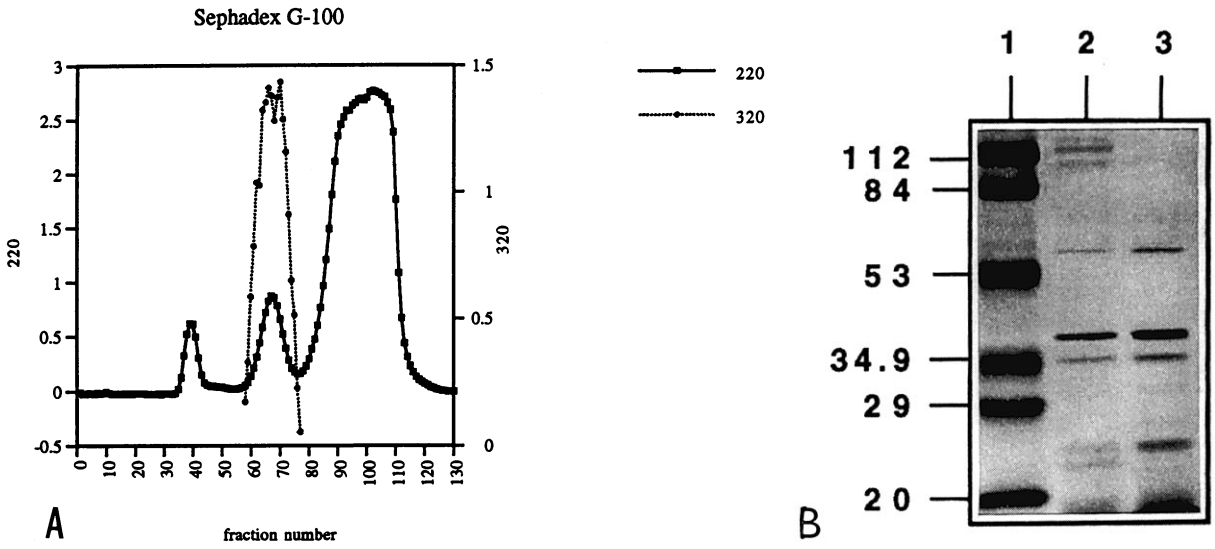

Figure 1. Separation of proteins from *Bacillus cereus* crude culture filtrate by: (A) Filtration through a Sephadex G-100 column. (B) SDS-PAGE: lane 1, molecular-weight markers; lane 2, before gel filtration through a Sephadex G-100 column; lane 3, after gel filtration.

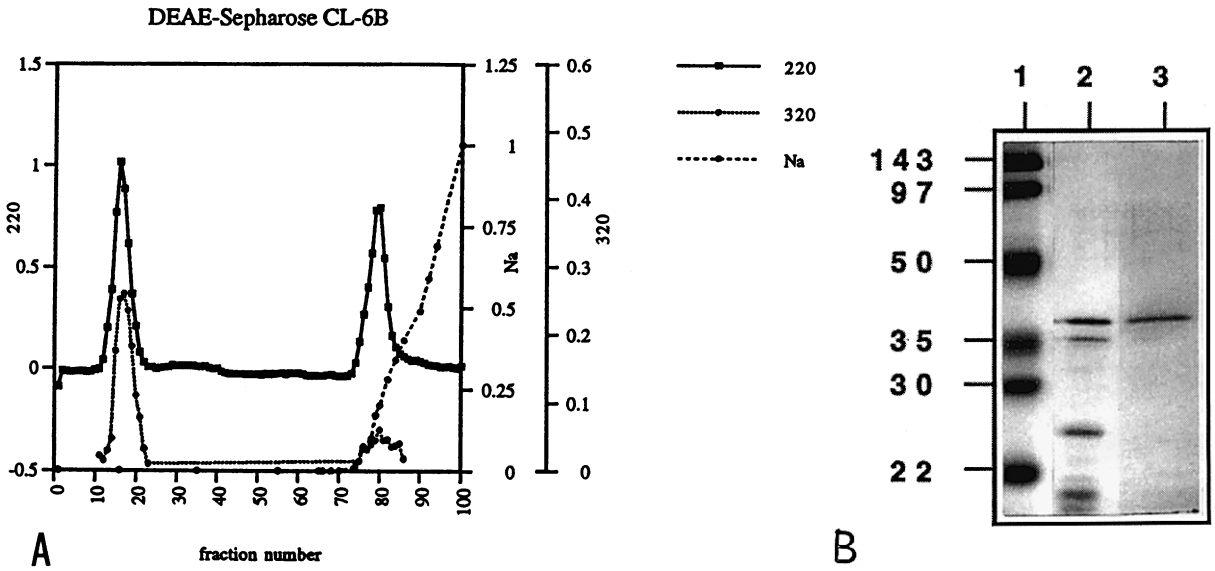

Figure 2. Bacillus cereus proteins separated on a DEAE-Sepharose column. (A) Profile of proteins eluted from the anion-exchange column with different concentrations of NaCl, as measured at 220 nm; collagenolytic activity was measured at 320 nm. (B) SDS-PAGE profile: Fractions expressing collagenolytic activity were collected from the anion-exchange column, combined, and a 5-mg aliquot loaded onto an SDS-PAGE (lane 2); the 42.8-kDa band was electro-eluted and 5 mg were re-electrophoresed (lane 3). Lane 1, molecular-weight markers.

activities in the collagen and NB media were 1.10 and 0.07, respectively. Maximum enzyme production was recorded when the culture was shaken (200 rpm) for 24 h at 32 °C in flasks. Zymography of the culture filtrate revealed various gelatinase-like proteins (data not shown).

The first purification step, through a Sephadex G-100 gel filtration column, yielded three protein-related peaks at 220 nm. Collagenolytic activity was only

detected in peak 2 (Figure 1A). SDS-PAGE analysis of the pre- and post-purified proteins revealed several protein impurities in addition to the main 42.8-kDa band corresponding to the collagenase protein (Figure 1B). Therefore, the fraction expressing collagenolytic activity was further purified through a DEAE-Sepharose CL-6B (anion-exchange) column: collagenase activity was detected only in the non-adsorbed fractions (Figure 2A). Figure 3 describes the enzymatic activity before and after separation by ion-exchange chromatography. Several protease activities were detected before separation (lane 2), but only one of these was heat-stable (lane 3). The specific activity was further demonstrated after ion-exchange purification (lanes 4, 5) and was found to be collagenolytic (Figure 2A). The fraction in which no collagenolytic activity was detected contained mainly proteolytic activities (Figure 3, lanes 6, 7). The single band which exhibited collagenolytic activity (Figure 3 lanes 3, 4), was consistent with the single purified band appearing on the SDS-PAGE (Figure 2B, lane 3). Table 1 summarizes the characteristics of the enzyme at each stage of its purification.

Purification of the extracted crude enzyme increased its specific activity from 1.0 to 2.5 U/ml, i.e. purification was 2.5-fold, but the percentage of activity recovery was very low (Table 1).

Enzyme characteristics

The molecular mass of the denatured enzyme as determined by SDS-PAGE was 42.8 kDa (Figure 2B, lanes 2, 3).

Optimal enzymatic activity was recorded in a pH range of 5.4 to 8.2. From pH 8.95 to 9.3, activity decreased by 50%; below pH 5.4 and from pH 9.4 to pH 10.2, no collagenolytic activity was found. Enzymatic activity was stable from 4 to 40 °C; between 55 and 60 °C, activity decreased by 50% (data not shown).

Digestion of BSA by collagenase

BSA was partially digested by *B. cereus* collagenase after 10 min (Figure 4, lane 2); complete digestion was achieved within 18 h (Figure 4, lane 5). However, the commercial collagenase from *C. histolyticum* did not exhibit any proteolytic activity, even after 18 h (Figure 4, lane 6).

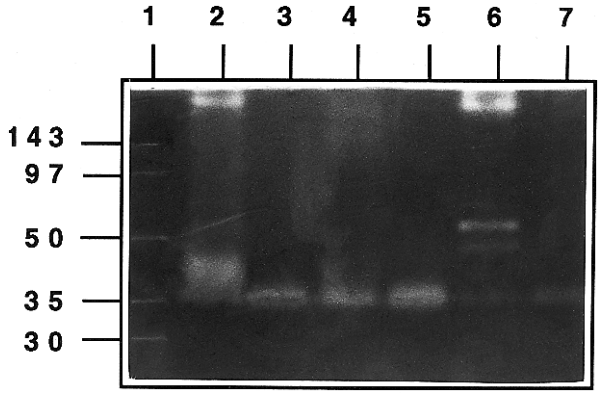

Figure 3. Zymography of the twice-purified *Bacillus cereus* enzyme: lane 1, molecular-weight markers; lanes 2 and 3, respectively, non-boiled and boiled filtrate before anion-exchange separation; lanes 4 and 5, respectively, non-boiled and boiled samples of peak 1 (see Figure 2A), after separation by anion-exchange chromatography; lanes 6 and 7, respectively, non-boiled and boiled samples of peak 2 (see Figure 2A).

Table 1. Characterization of the *Bacillus cereus* collagenase/protease at each purification step

Fraction	Crude filtrate	Gel filtration	Anion exchange
Volume (ml)	80.0	4.5	2.0
Protein concn (mg/ml)	206	173	70.6
Total protein (mg)	16.5	0.77	0.14
Collagenase activity (U/ml)	206	315	175
Total activity (U)	16500	1417	350
Specific activity (U/mg)	1000	1820	2500
Protein recovery (%)	100	4.7	0.85
Activity recovery (%)	100	8.56	2.12
Purification (fold)	1.0	1.82	2.5

Effect on cuticular proteins extracted from M. javanica

I. Separation of M. javanica *J2 collagenic-protein extracts by SDS-PAGE*

A typical SDS-PAGE pattern of the collagenic proteins extracted with β-mercaptoethanol is presented in Figure 5, lane 2. The major polypeptides were at 32, 35,

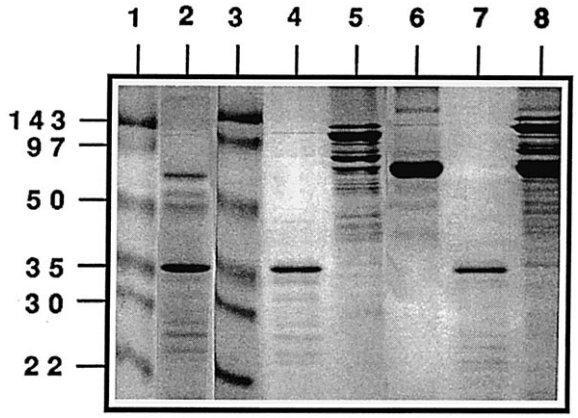

Figure 4. SDS-PAGE profile of bovine serum albumin (lane 6) digested with *Bacillus cereus* collagenase for 10 min (lane 2) or 18 h (lane 7), or digested with *Clostridium histolyticum* collagenase for 18 h (lane 8); lane 4, protein profile of the *B. cereus*-purified enzyme; lane 5, protein profile of the *C. histolyticum* collagenase. Lanes 1 and 3 are molecular-weight markers.

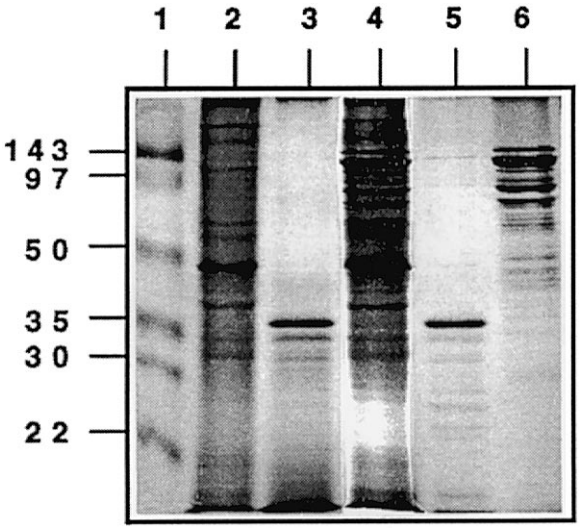

Figure 5. SDS-PAGE profile of soluble β-mercaptoethanol-extracted proteins from cuticles of *Meloidogyne javanica* second-stage juveniles, incubated for 10 min with two collagenases. Lane 1, molecular-weight markers; lane 2, non-treated extracted proteins; lane 3, after treatment with the *Bacillus cereus*-purified enzyme; lane 4, after treatment with a commercial *Clostridium histolyticum* collagenase; lane 5, protein profile of the *B. cereus*-purified enzyme; lane 6, protein profile of the *C. histolyticum* collagenase.

39, 46, 51, 56, 68, 74, 81, 85, 91, 98, 105, 123, 138 and 141 kDa.

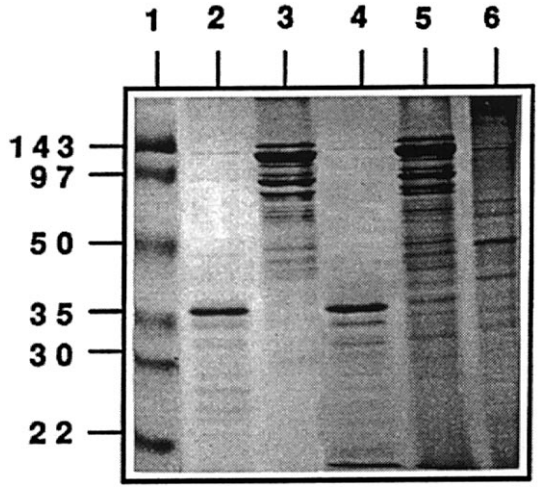

Figure 6. SDS-PAGE profile of soluble β-mercaptoethanol-extracted proteins from cuticles of *Meloidogyne javanica* second-stage juveniles (lane 6), incubated for 120 min with the *Bacillus cereus*-purified enzyme (lane 4) or with a commercial *Clostridium histolyticum* collagenase (lane 5); lane 3, after treatment, for 120 min, with a commercial *Clostridium histolyticum* collagenase; lane 2, protein profile of the *B. cereus*-purified enzyme; lane 6, protein profile of the *C. histolyticum* collagenase; lane 1, molecular-weight markers.

II. Digestion of M. javanica *cuticle by collagenase*

The *B. cereus*-isolated collagenase completely digested the β-mercaptoethanol-soluble proteins extracted from *M. javanica* J2 cuticle within 10 min (Figure 5, lane 3). During that time, the pattern of collagenic proteins was hardly affected by the commercial collagenase from *C. histolyticum* (Figure 5, lane 4). Nearly complete digestion of the *M. javanica* cuticle proteins by the *C. histolyticum* collagenase took over 2 h (Figure 6, lane 3). The collagen of kangaroo origin was completely digested by both *B. cereus* and *C. histolyticum* collagenases within 18 h (Figure 7, lanes 6 and 7, respectively). However, during that time, the rat-tail collagen was not digested by either *B. cereus* or *C. histolyticum* collagenases (Figure 7, lanes 3 and 4, respectively).

Discussion

Keil-Dlouba et al. (1976) and Merkel and Dreisbach (1978) found that the presence of a collagen(s) or its decomposition products in the growth medium induces collagenase secretion in measurable amounts. Moreover, this collagenase was relatively free of non-

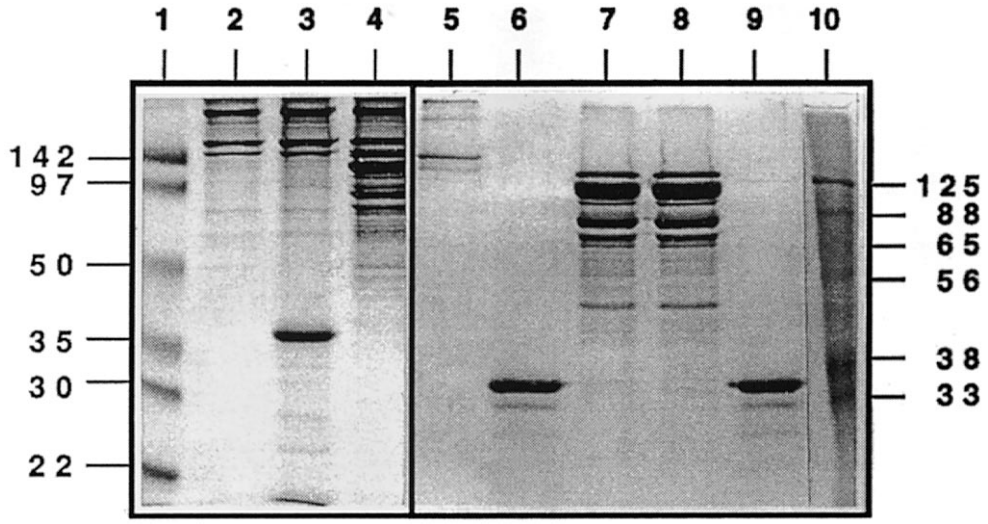

Figure 7. SDS-PAGE profile of commercial rat tail (lane 2) and kangaroo (lane 5) collagens, incubated for 18 h with the *Bacillus cereus*-purified enzyme (lanes 3 and 6, respectively) and with a commercial *Clostridium histolyticum* collagenase (lanes 4 and 7, respectively); lanes 8 and 9 represent the commercial *C. histolyticum* and *B. cereus*-purified collagenases, respectively, and lanes 1, and 10 are molecular-weight markers.

specific proteases. The results reported here concur with those findings: the overall amount of protein secreted into the culture filtrate was higher when *B. cereus* was grown in NB, but it contained a higher amount of non-specific proteases (Figure 2). Accordingly, enzyme activity was relatively higher when *B. cereus* was grown on collagen medium.

An activity gel of the proteins secreted into the culture filtrate of collagen-grown *B. cereus* revealed several enzymes capable of decomposing gelatine (data not shown); however gelatine, a denatured form of collagen, can be digested by any non-specific protease, in addition to collagenase. As shown in Figure 2B lane 3, the enzyme migrated as a single sharp band with no trace of contaminants. At this stage of the enzyme purification process, only 2% of the enzyme activity was retained, a phenomenon similar to that found by Sasagawa et al. (1993).

Significant differences in collagenase size have been reported in prokaryotic cells: the molecular mass of a collagenase *of Pseudomonas* origin was 17.5 kDa (Carrick and Berk, 1975), whereas that of the enzyme of *Vibrio* origin was 100 kDa (Merkel and Dreisbach, 1978). In this work, the size of the enzyme was estimated at 42.8 kDa by SDS-PAGE (Figure 2B). Similar to a report by Kawahara et al. (1993), *B. cereus* collagenase revealed activity at acidic pH. Its overall optimal activity range was quite broad however, from pH 5.4 to 8.2 in a Tris-maleate buffer. A similarly wide range of activity (6.2 to 7.8) has been reported by Mandl et al. (1958) for *C. histolyticum* collagenase in Tris and phosphate buffers. These authors also found that below pH 5.0 this enzyme suffers an irreversible loss of activity. *Clostridium welchii* collagenase exhibits an irreversible loss of activity at pHs above 10.4 (Bidwell, 1949). In this work, an irreversible loss of *B. cereus* collagenase activity occurred at pHs below pH 5.4 and from pH 9.4 to at least 10.2. A 50% decrease in activity was measured between pHs 8.95 and 9.3.

The cuticular proteins isolated from *Meloidogyne incognita* (Reddigari et al., 1986), and from *M. javanica* in this work (Figure 5, lane 2), were digested by *C. histolyticum* collagenase (Figures 5 and 6, lanes 4 and 3, respectively), suggesting that these proteins are collagenic. Both studies also showed that collagenase of *Clostridium* origin does not digest BSA (Figure 4, lane 6), leading to the conclusion that this enzyme lacks non-specific proteolytic activity. On the other hand, *B. cereus*-isolated-collagenase digested BSA (Figure 4, lanes 2 and 5), as well as the collagenic components of *M. javanica*, suggesting that this enzyme possesses both collagenolytic and proteolytic activities.

The *B. cereus*-isolated enzyme exhibited some measure of specificity: although it was isolated from bacteria grown on collagen type I originating from bovine Achilles tendon, it could digest collagens from J2 cuticles (Figures 5 and 6, lanes 3 and 2, respectively) and from kangaroo (Figure 7, lane 6), but not from

rat tail (Figure 7, lane 3). Moreover, *B. cereus* collagenase's digestion of BSA within 10 min revealed its strong proteolytic activity (Figure 4, lane 2). This explains why collagens of nematode origin were only partially digested by *C. histolyticum* after 120 min, whereas the *B. cereus* collagenase completed their digestion within 10 min (Figure 5, lane 3).

Information on root-knot nematode cuticular collagens is scarce; the root-knot nematode cuticle is composed of several collagenic/proteinaceous layers (Kingston, 1991; Ray et al., 1996). Recently, polyclonal antiserum was raised against the major 76-kDa collagen protein extracted from cuticles of *M. incognita* adult females and then used in immunogold electron microscopy to localize collagen in the cuticles of different life stages (Ray et al., 1996). In vermiform preparasitic J2, which have distinct three-zoned cuticles, labelling occurred only within the cortical zone; however, in the adult form, which has a homogeneously structured cuticle, labelling was uniformly distributed over the entire width of the internal structure of the cuticle (Ray et al., 1996). Ray et al.'s (1996) findings, as well as previous reports by Reddigari et al. (1986) and Kingston (1991), that collagens are the major component of root-knot nematode cuticles, suggest that disruption of cuticular collagens may affect the nematode cuticle, resulting in disruption of the nematode's life cycle. Thus the *B.cereus* collagenase/protease may prove to be an efficient agent for attacking and disrupting this nematode's cuticular collagens in both pre- and post-parasitic forms, resulting in a potentially effective way of controlling root-knot nematodes.

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