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A Novel, Symbiotic Bacterium Isolated from Marine Shipworm Secretes Proteolytic Activity

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Abstract. Proteolytic activity was identified in cultures of the marine shipworm bacterium by monitoring of the release of acid-soluble azopeptides from azocasein. Activity was predominantly extracellular (>80%), and its production was coincident with logarithmic cell growth. The protease(s) appeared to be constitutive, since it was present even when the bacterium was grown under nitrogen-fixing conditions. However, activity was stimulated up to 8.6-fold by the addition of complex nitrogen (casein, amino acids) to the growth medium. Maximum activity was observed at 40°C and between pH 6.5 and 9.0. Relatively low concentrations (0.1 m*M*) of phenylmethylsulfonyl fluoride (PMSF) abolished activity, indicative of a serine protease(s).

Marine bivalves of the family Teredinidae (commonly referred to as shipworms) are exclusively wood-boring mollusks and have been rather extensively studied in an effort to protect wooden structures in marine environments [12]. Although it is generally agreed that the shipworms digest eroded wood fragments liberated during the boring process, much controversy exists as to the source of enzymes necessary for cellulose digestion. Waterbury et al. [13] isolated a nitrogen-fixing, cellulolytic bacterium from a specialized gland of the shipworm, known as the gland of Deshayes. The gland lines the afferent branchial vein and opens into the esophagus, somewhat analogous to a salivary gland [12], and harbors a dense, pure culture of the bacterium [2]. It was postulated that this bacterium is a primary source of cellulose-digestive enzymes [13]. Indeed, subsequent investigations determined that the shipworm bacterium secretes considerable endoglucolytic activity [9], and recently an extracellular endoglucanase from the bacterium was isolated and characterized [8].

Shipworms also possess a functional gill (ctenidium) [12] and are capable of opportunistic filter-feeding on proteinaceous material, such as plankton and detritus. Since filtered material ultimately passes through the esophagus, it seemed possible that the shipworm bacterium might also be a source of proteolytic enzymes. In this study, we report and partially characterize extracellular proteolytic activity from shipworm bacterium cultures.

Materials and Methods

Cell culture. Bacterial isolates (Woods Hole Oceanographic Institution strain number T8301) from *Psiloteredo healdi* shipworm were a generous gift of Dr. John Waterbury. Cell growth was conducted at 25°C with 50 ml of culture medium in 250-ml Erlenmeyer flasks as previously described [7]. The carbon source was 0.5% Sigmacell 100. Cultures not supplemented with complex nitrogen were incubated statically; this maintained microaerobic conditions necessary for nitrogen fixation. Cultures supplemented with complex nitrogen sources were aerated by shaking at 125 rpm. The amino acid supplement indicated in the text was an equal weight amount of leucine, serine, methionine, aspartate and lysine.

Assays. Proteolytic activity was monitored with azocasein for the substrate, as described elsehwere [4]. Briefly, the assay contained 0.5 ml of 0.8% azocasein in 100 m*M* potassium phosphate buffer (pH 7) and 0.5 ml of the enzyme source. The reaction medium was incubated in 1.8-ml microcentrifuge tubes for 3 h at 25°C. The reaction was halted by addition of 0.5 ml of cold 1.5 *M* $HClO_4$ to each tube, which was then held on ice for 30 min. Precipitated protein was removed by centrifugation (13,000 *g*, 5 min), and a 1.0-ml aliquot of supernatant was combined with an equal volume of 1 *N* NaOH. The concentration of acid-soluble azopeptides was determined optically at 440 nm, with 1.00 optical density unit equal to 320 $\mu g \cdot ml^{-1}$. All assays were conducted at least in duplicate.

Cell protein (defined as material that pellets at 13,000 *g* for 5 min) was determined essentially by the method of Lowry et al.

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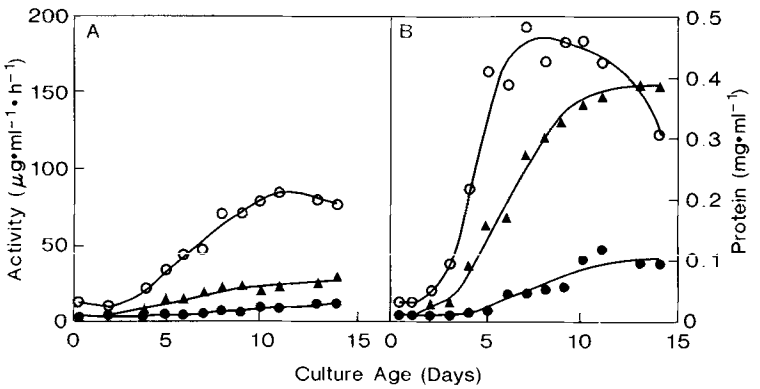

Fig. 1. Growth of shipworm bacterium and production of proteolytic activity. (A) Cultures grown under nitrogen-fixing conditions. (B) Cultures supplemented with 0.05% ammonium chloride. Symbols: (○) cell protein; (●) cell-free protein; (▲) proteolytic activity. All points represent duplicate cultures.

Table 1. Effect of added nitrogen source on proteolytic activity

Nitrogen source	Cell protein (mg · ml^{-1})	Proteolytic activity (units · ml^{-1})[a]	Relative activity (units · mg cell protein^{-1})	Normalized activity
None	0.18	16	89	1.0
0.05% Ammonium chloride	0.42	149	355	4.0
0.05% Casein	0.40	214	535	6.0
0.05% Amino acids	0.39	257	659	7.4
0.025% Casein plus 0.025% ammonium chloride	0.44	261	593	6.7
0.025% Casein plus 0.025% amino acids	0.48	368	767	8.6

[a] Units represent μg azocasein digested per hour.

[11], except that after addition of alkaline copper, samples were incubated at 80°C for 30 min to aid cell disruption. Cell-free protein (the supernatant from the above centrifugation) was determined by the procedure of Bradford [3].

Chemicals. Ethylenediaminetetraacetic acid (EDTA), thimerosal, iodoacetamide, *p*-tosyl-L-lysine chloromethyl ketone (TLCK), N-tosyl-L-phenylalanine chloromethyl ketone (TPCK), trypsin inhibitor I-S, phenylmethylsulfonyl fluoride (PMSF), dithiothreitol, azocasein, casein, and individual amino acids were from Sigma Chemical Co. All other chemicals were of reagent grade.

Results

Proteolytic activity as a function of culture age. The shipworm bacterium was grown under conditions conductive to, as well as under conditions that prohibit, nitrogen-fixation. Proteolytic activity as a function of culture age was monitored by azocasein digestion. As expected, the culture supplemented with combined nitrogen (ammonium chloride) grew faster and to a greater extent than did the culture forced to obtain its combined nitrogen via nitrogen fixation (Fig. 1). Both cultures exhibited significant proteolytic activity, which coincided with logarithmic cell growth. However, the ratio of proteolytic activity to cell density was higher in the culture supplemented with ammonium chloride.

Effect of nitrogen source on production of proteolytic activity. Different sources of combined nitrogen were examined for their effect on production of proteolytic activity. The extent of cell growth was not significantly affected by the source of supplemental nitrogen, although some variations in growth rate and initial lag phase were observed (not shown). The data presented in Table 1 summarize proteolytic activity and cell protein yields for cultures in late logarithmic growth phase. As noted above, proteolytic activity per mg cell protein was 4.0-fold higher in cultures supplemented with ammonium chloride than in nitrogen-fixing cultures. Substituting protein (casein) or amino acids for ammonium chloride further enhanced proteolytic activity relative to cell protein. The greatest increase

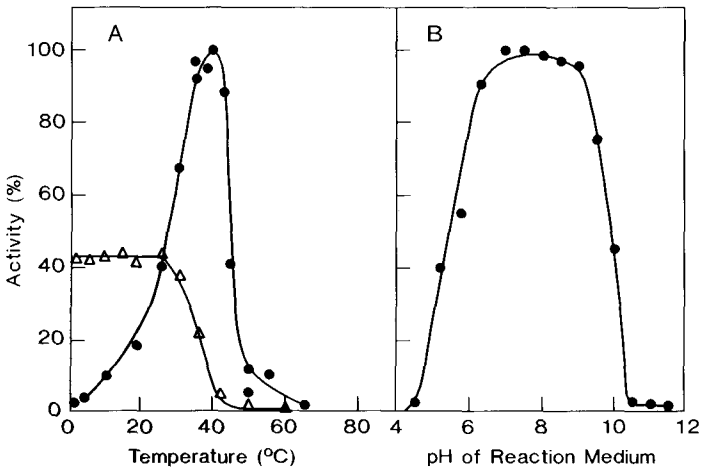

Fig. 2. Effects of temperature and pH on cell-free proteolytic activity. (A) For thermal activity (●), enzyme reactions were conducted for 1 h at indicated temperatures. For thermal stability (△), cell-free culture medium was pre-incubated at indicated temperatures for 1 h and then reacted with azocasein for 3 h at 25°C. (B) Reactions were conducted at indicated pH values with a buffer of 50 m*M* sodium citrate, 50 m*M* potassium phosphate, 50 m*M* tris(hydroxymethyl)aminomethane, and 50 m*M* glycine.

Table 2. Cellular location of activity

Preparation	Proteolytic activity (units · ml^{-1})[a]	Activity (%)
Intact whole culture	173	100
Cell-free culture medium	146	84
Intact cell pellet[b]	41	24
French-pressed whole culture	158	91
French-pressed cell pellet[b]	44	25

[a] Units represent μg azocasein digested per hour.
[b] Cell pellets resuspended in basal salts buffer [6] to original culture volume.

in activity per mg cell protein was seen in cultures supplemented with both casein and amino acids; these cultures were stimulated 8.6-fold compared with nitrogen-fixing cultures. Interestingly, cell growth was absent when casein was provided as both an energy and nitrogen source (not shown).

Cellular location of activity. More than 80% of the original proteolytic activity present in a late logarithmic growth phase culture (plus NH_4Cl) remained in the cell-free supernatant after centrifugation (13,000 *g*, 5 min); this suggests that the activity is due to a secreted enzyme(s) (Table 2). The proportion of activity present in the extracellular fluid did not change with the stage of culture growth (not shown). Similar results were obtained for cultures grown with other nitrogen sources. Disrupting the shipworm bacterium in a French pressure cell did not result in increased activity (Table 2), further indicative that the protease(s) was extracellular in nature.

Effect of temperature and pH on activity. Cell-free culture medium was utilized to further characterize some of the physiological properties of the proteolytic activity. Maximum activity was observed at 40°C, but the enzyme(s) was unstable at temperatures exceeding 25°C (Fig. 2A). Freezing had little effect on activity. A broad activity maximum was observed between pH 6.5 and pH 9.0, whereas >50% of maximal activity occurred from pH 5.2 to pH 9.9 (Fig. 2B).

Inhibitors. Several compounds known to inhibit various proteases were examined for their effect on the activity of the shipworm bacterium (Table 3). The metalloenzyme inhibitor, EDTA, reduced activity. However, this could be a nonspecific effect because rather high concentrations of EDTA were necessary to achieve significant inhibition. Thiol proteases can be inhibited by thimerosal and iodoacetamide [14], but only the former was inhibitory at high concentrations. In addition, reducing agents such as cysteine and dithiothreitol did not stimulate proteolytic activity (not shown). PMSF, which reacts with hydroxyl moieties of active site serines [14], abolished proteolytic activity at low concentrations, strongly suggestive that the active site of the enzyme(s) contains a serine residue. However, TLCK and TPCK, which inhibit serine proteases with chymotrypsin- and trypsin-like activities [14], were ineffective. Likewise, trypsin inhibitor I-S was not inhibitory. Therefore, the shipworm bacterium enzyme(s), although probably a serine protease(s), apparently does not possess an active site similar to that of trypsin or chymotrypsin.

Table 3. Effect of potential inhibitors on activity[a]

Compound	Final concentration (m*M*)	Activity (%)
None	—	100
EDTA[b]	10	35
	1.0	80
Thimerosal[b]	1.0	64
	0.1	95
Iodoacetamide[b]	1.0	91
PMSF[c]	1.0	6
	0.1	5
TLCK[c]	1.0	95
TPCK[c]	1.0	92
Trypsin inhibitor I-S[b]	100[d]	89

[a] Activity determined on cell-free medium from 0.05% ammonium chloride-supplemented culture.
[b] Added as 0.1 ml of 100 m*M* tris(hydroxymethyl)aminomethane buffer (pH 8) to enzyme source and preincubated for 15 min.
[c] Added as 0.1 ml of ethanol to enzyme source and preincubated for 15 min. Inhibition by ethanol alone was not observed.
[d] Concentration is $\mu g \cdot ml^{-1}$.

Discussion

The shipworm bacterium apparently produces a constitutive level of proteolytic activity even in the absence of environmental nitrogenous compounds, as evidenced by the presence of activity during growth conditions conducive to nitrogen fixation (Fig. 1). This activity was stimulated by the addition of combined nitrogen sources to the medium (Table 1), suggestive of the possibility that a regulatory mechanism exists. At optimal temperature (40°C, Fig. 2A) and pH (6.5 to 9.0, Fig. 2B), the activity in a culture supplemented with casein and amino acids approached 1 mg azocasein digested $\cdot ml^{-1} \cdot hr^{-1}$. Interestingly, this is quite similar to the optimized enzymatic activities reported for the important proteolytic bacteria of the rumen (*Bacteroides amylophilus, Bacteroides ruminicola,* and *Butyrivibrio fibrisolvens*) [2, 4, 10], whose symbiotic environment is somewhat analogous to that of the shipworm bacterium.

The protease(s) appears to be secreted by the bacterium because less than 20% of the activity was found to be intracellular and/or associated with the cell wall (Table 2). It is curious that a bacterium capable of nitrogen fixation (Fig. 1) and incapable of growth on protein (casein) should secrete such significant levels of proteolytic activity. Furthermore, this activity does not seem to be crucial to cell maintenance, since the bacterium grows optimally at 35°C [7], whereas the proteolytic activity is unstable above 25°C (Fig. 2). These observations suggest that the protease(s) may play a role in the symbiotic relationship between shipworm and bacterium. In other microbial systems it is known that proteolytic activity enhances the hydrolysis of cellulose from woody plant materials [5, 6]. This could likewise be the case for the proteolytic activity described in this report, because the bacterium ostensibly degrades cellulose for the host shipworm [2, 5, 6]. Alternatively, the protease(s) could be directly involved in protein digestion when the shipworm is filter-feeding on proteinaceous material.

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