Further Characterization of Renibacterium salmoninarum Extracellular Products

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Renibacterium salmoninarum, the agent of bacterial kidney disease in salmonids, releases high concentrations of extracellular protein in tissues of infected fish. The extracellular protein consists almost entirely of a 57-kDa protein and derivatives of degradation and aggregation of the same molecule. The 57-kDa protein and its derivatives were fractionated into defined ranges of molecular mass. Separated fractions continued to produce degradation and aggregation products. One-dimensional electrophoretic separation of extracellular protein revealed a number of proteolytically active bands from >100 to approximately 18 kDa associated with various 57-kDa protein derivatives in the different molecular mass fractions. Two-dimensional separation of extracellular protein showed that continued degradation and aggregation, similar both in location and behavior to some of the 57-kDa protein derivatives, was also displayed by the proteolytically active bands after their separation. Effects of reducing agents and sulfhydryl group proteinase inhibitors indicated a common mechanism for the proteolytically active polypeptides characteristic of a thiol proteinase. The results suggested that the 57-kDa protein and some of its derivatives undergo autolytic cleavage, releasing a proteolytically active polypeptide(s) of at least 18 kDa. Soluble polysaccharide-like material also was detected in extracellular products and tissue from infected fish. Antiserum to the polysaccharide-like material cross-reacted with polypeptide(s) of at least 18 kDa. Soluble polysaccharide-like material also was detected in extracellular products and tissue from infected fish. Antiserum to the polysaccharide-like material cross-reacted with polypeptide(s) of at least 18 kDa. Soluble polysaccharide-like material also was detected in extracellular products and tissue from infected fish. Antiserum to the polysaccharide-like material cross-reacted with polypeptide(s) of at least 18 kDa. Soluble polysaccharide-like material also was detected in extracellular products and tissue from infected fish. Antiserum to the polysaccharide-like material cross-reacted with polypeptide(s) of at least 18 kDa. Soluble polysaccharide-like material also was detected in extracellular products and tissue from infected fish. Antiserum to the polysaccharide-like material cross-reacted with polypeptide(s) of at least 18 kDa. Soluble polysaccharide-like material also was detected in extracellular products and tissue from infected fish. Antiserum to the polysaccharide-like material cross-reacted with polypeptide(s) of at least 18 kDa. Soluble polysaccharide-like material also was detected in extracellular products and tissue from infected fish. Antiserum to the polysaccharide-like material cross-reacted with polypeptide(s) of at least 18 kDa. Soluble polysaccharide-like material also was detected in extracellular products and tissue from infected fish. Antiserum to the polysaccharide-like material cross-reacted with polypeptide(s) of at least 18 kDa. Soluble polysaccharide-like material also was detected in extracellular products and tissue from infected fish. Antiserum to the polysaccharide-like material cross-reacted with polypeptide(s) of at least 18 kDa. Due to the high concentrations of p57 and its degradation products in tissues of infected fish and the involvement of p57 in the virulence of R. salmoninarum, further investigation to characterize the ECP was undertaken.

MATERIALS AND METHODS

Bacterial strains and cultivation. The Canadian east coast R. salmoninarum F91 strain was isolated from Atlantic salmon (Salmo salar L.) as reported previously (17). The west coast R. salmoninarum WC strain was isolated from chinook salmon (Oncorhynchus tshawytscha) and kindly provided by J. Brackett. Bacteria were cultivated on selective kidney disease medium agar (1) for 3 to 4 weeks at 15°C, and the ECP were harvested in phosphate-buffered saline (PBS) at 4°C as reported previously (12) and stored at −20°C until processed.

Sources (infected Atlantic salmon) and cultivation of other bacterial strains used, i.e., Aeromonas salmonicida Aso92, A. salmonicida Ast5, Pseudomonas fluorescens F, and Vibrio ordali B, were as reported elsewhere (11).

Fractionation of R. salmoninarum extracellular polypeptides. ECP preparations were concentrated (5 to 6 mg of protein ml−1) with Microcon B-15 macroporous concentrators (Amicon, Inc., Beverly, Mass.), and aliquots (approximately 1,800 µg of protein) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at room temperature as described elsewhere (27) with a Mini-Protean II gel electrophoresis system (Bio-Rad Laboratories, Ltd., Hercules, Calif.) in a single wide well. The polypeptides were transblotted to Immobilon membranes (Millipore Corp., Bedford, Mass.) as described by Szewczyk and Summers (23). The membrane was wetted with 95% (vol/vol) methanol prior to equilibration in transfer buffer. Electrophoretic transfer was at 20 V for 1 h in a semidry electrophoretic transfer cell (Bio-Rad). After transfer, the membrane was stained with 0.2% (wt/vol) amido black to locate polypeptides and destained with deionized distilled H2O. Horizontal strips containing polypeptide bands were excised from the Immobilon membrane and eluted by washing with 1 ml of 1% (vol/vol) Triton X-100 and 2% (vol/vol) SDS. The eluates (fractions) were dialyzed overnight at 4°C against deionized distilled H2O. Western blotting (immunoblotting) of the isolated fractions (1 ml) was performed with 100-µl aliquots of fractions 1, 2, 3, and 6 and with 50-µl aliquots of fractions 4 and 5 (see Fig. 1) as described previously (13), except that the first binding agent was a monoclonal anti-p57 protein antibody (4D3; Diagxotics, Inc., Wilton, Conn.) solution of 30 µl added to 10 ml of 10 mM Tris-HCl-150 mM NaCl-0.5% (vol/vol) Tween 20 (pH 7.5; TTBS) and the second binding agent was...
biotinylated goat anti-mouse immunoglobulin (Amersham Corp., Arlington Heights, IL) solution of 20 μl added to 10 ml of TTBS.

**PAGE.** For one-dimensional gelatin-SDS-PAGE (G-PAGE), the gels were prepared as described for SDS-PAGE except that 0.1% (wt/vol) gelatin was included in the lower gel and aliquots of ECP were added directly to sample buffer (16) prior to electrophoresis. Subsequent to electrophoresis, the gels were treated as described elsewhere (22) to detect proteolytic activity.

For two-dimensional G-PAGE, aliquots of ECP in sample buffer were separated as described previously (12), except that gel strips containing the separated polypeptides from the one-dimensional SDS-PAGE gels were incubated for 1 h at 20°C in 2% (vol/vol) Triton X-100 and then for 4 h in PBS and 10 min in sample buffer. Subsequently, the gel strip was inserted on top of the two-dimensional stacking gel (0.5 cm) without the aid of molten agarose, and the running gel used contained 0.1% (wt/vol) gelatin. Proteolytic activity was detected as described above.

**Two-dimensional Western blots of R. salmoninarum ECP.** Two-dimensional SDS-PAGE gels were run as described for two-dimensional G-PAGE gels except that 0.1% (wt/vol) gelatin was omitted. Western blotting of the two-dimensional gel was done as reported previously (12).

**Effects of DTT and proteinase inhibitors on protein digestion by the R. salmoninarum ECP.** Dithiothreitol (DTT) and proteinase inhibitors were dissolved in PBS and added to give the final concentrations indicated (see Fig. 4 and Results) to aliquots of the ECP in PBS (1 ml total volume). PMSF was first dissolved in isopropanol and then diluted in PBS. The mixtures were left for 10 min at room temperature prior to the addition of bovine serum albumin (BSA; 10 mg in 1 ml of PBS) and incubation at 35°C. Aliquots of the incubations (where indicated at the start of incubation were processed by SDS-PAGE. Digestion of BSA was visualized by staining the gels with Coomassie blue (LKB application note 321). Also, DTT and inhibitors were added to aliquots of the ECP in PBS and incubated at 35°C. Samples were removed at the times indicated, precipitated with trichloroacetic acid (10% [vol/vol] final concentration), and samples equivalent to 20 μg of BSA at the start of incubation were applied to SDS-PAGE. Gelatinolytic activity of the ECP was determined by the appearance of the characteristic yellow bands (Bio-Rad application note 321). DTT and proteinase inhibitors were dissolved in PBS containing the final concentrations indicated (see Fig. 5) at 37°C in PBS containing pronase (final concentration, 2 mg ml−1; Calbiochem-Behring Corp., La Jolla, Calif.). Samples were removed at the designated times, precipitated with trichloroacetic acid, and examined by Western blotting. The Western blots were developed with the antiserum prepared prior to two-dimensional electrophoretic separation. The two-dimensional G-PAGE profiles of the proteolytic bands (Fig. 2A, lane 2) as reported previously (22). However, in several ECP preparations, a variable number of additional proteolytic bands from >100 kDa to approximately 18 kDa were also detected (Fig. 2A, lane 1). The location of proteolytic bands (e.g., at approximately >57, 57, 37, 33, and 18 to 22 kDa) was similar to that of some of the major p57 derivatives detected on Western blots (e.g., Fig. 1, lanes 2 to 7). The ECP polypeptides were subjected to SDS-PAGE, and gel strips containing the separated polypeptides were incubated (4 h at 20°C) prior to two-dimensional electrophoretic separation. The two-dimensional G-PAGE profiles of the proteolytic bands (Fig. 3A) resembled two-dimensional Western blot profiles of the p57 derivatives (Fig. 3B) in that some proteolytic activity was observed to migrate both below and above the expected molecular mass positions (diagonal) of the proteolytic bands on the two-dimensional gel.
Effects of inhibitors and reducing agents on the *R. salmoninarum* extracellular proteolytic activity. Digestion of the autologous p57 protein in the *R. salmoninarum* ECP was reported to be inhibited by 1 to 2 mM PMSF (6, 12, 22). Also, digestion of unrelated proteins such as BSA by the ECP proteolytic activity was reported to require or was stimulated by the addition of SDS or DTT (22). The connection between these observations and the multiple proteolytic bands detected in the ECP was investigated.

For both *R. salmoninarum* strains, addition of PMSF to the ECP inhibited the activity of all proteolytic bands digesting gelatin on G-PAGE gels, and higher concentrations of PMSF (6 mM) were required to inhibit the proteolytic activity against gelatin in ECP preparations containing DTT (10 mM) (Fig. 2B). Similarly, stimulation of BSA digestion by the ECP proteolytic activity in the presence of DTT (10 mM) (Fig. 4A, lane 7). Similar effects on BSA digestion were observed when other sulfhydryl group proteinase inhibitors, including iodoacetate (10 mM), iodoacetamide (10 mM), N-ethylmaleimide (10 mM), and 2,2-dipyridyl disulfide (0.25 mM), replaced PMSF (data not shown). Auto-digestion of the autologous p57 protein derivatives in the ECP was stimulated by DTT (Fig. 4B) and inhibited by the sulfhydryl group proteinase inhibitors mentioned above in a similar manner (data not shown).

*R. salmoninarum* extracellular polysaccharide-like material. To determine the nature of extra nonproteinaceous material in the ECP from both strains, Western blots of pronase-digested ECP were developed with rabbit polyclonal antisera prepared against either the whole ECP or *R. salmoninarum* cells, Western blots of pronase-digested kidney tissue homogenates from infected Atlantic salmon (*S. salar*) revealed the presence of carbohydrate moieties detected in kidney tissue homogenates from fish without clinical signs of BKD. The presence of carbohydrate moieties associated with this polysaccharide-like material was detected
on Western blots (Fig. 5C) by use of a carbohydrate detection system (GlycoTrack; Oxford GlycoSystems). Furthermore, periodate treatment (28) to disrupt these carbohydrate moieties blocked the immunoreaction of the antiserum with the polysaccharide material (Fig. 6). Interestingly, this antiserum prepared against _R. salmoninarum_ cells boosted with protein-free cell lysate cross-reacted on Western blots with O polysaccharides in outer membrane lipopolysaccharide fractions from _A. salmonicida_ but not with O polysaccharides from other gram-negative bacteria tested, i.e., _V. ordalii_ or _P. fluorescens_ (Fig. 7). No cross-reaction of this antiserum with the _A. salmonicida_ extracellular or outer membrane proteins was detected (data not shown).

**DISCUSSION**

Polypeptides in the _R. salmoninarum_ ECP, consisting mainly of p57, its breakdown products, and aggregates, are digested by an autologous proteolytic activity (22). In the current study, the release of p57-derived fragments from separated ECP components suggested that proteolytic activity is present in or is a core component of many molecular mass fractions. The ability of PMSF to inhibit the generation of fragments within the separate fractions supported this suggestion. Interestingly, fractions containing p57 or its breakdown products also appeared to form larger-molecular-mass aggregates.

The use of one-dimensional G-PAGE to locate ECP proteolytic activity against gelatin produced variable results with different ECP preparations. Often, a broad band of proteolytic activity was detected at >100 kDa as reported previously (22). However, a variable number of additional proteolytic bands at positions as low as approximately 18 kDa were also often seen at locations occupied by some of the major p57 derivatives. The observation that some proteolytic activity migrated below and above the expected molecular mass positions for several of these proteolytic bands on two-dimensional G-PAGE gels im-

**FIG. 5.** Western blot detection of extracellular polysaccharide produced by _R. salmoninarum_. ECP (300 μg of protein ml⁻¹) from _R. salmoninarum_ F91 (A, B, and C) or kidney tissue homogenate (50 μl ml⁻¹) from Atlantic salmon (_S. salar_ L.) clinically infected with _R. salmoninarum_ (D and E) were digested with pronase (2 mg ml⁻¹) at 35°C. Samples equivalent to 8 (A) and 40 (B and C) μg of ECP protein or to 0.25 (D) and 0.5 (E) μl of kidney tissue at 0 h of incubation were applied to gels after 1 (lanes 1), 3 (lanes 2), 6 (lanes 3), and 24 (lanes 4) h of incubation with pronase. The Western blots of panels A and D were developed with polyclonal anti-whole _R. salmoninarum_ ECP antiserum, whereas blots of panels B and E were developed with polyclonal anti-pronase-digested cell lysate-boosted whole-cell antiserum. The blot of panel C was developed with a carbohydrate detection system (GlycoTrack; Oxford GlycoSystems). Prestained molecular mass markers as described in the legend to Fig. 1 are shown (B and C, lanes 5), and the position of the 57-kDa protein is indicated to the left of the figure. Native molecular mass markers reacting with the GlycoTrack system, including BSA (67 kDa), ovalbumin (43 kDa), and α-lactalbumin (14.4 kDa), are also shown (C, lane 6).

**FIG. 6.** Western blot of the effect of periodate oxidation on the immunodetection of the _R. salmoninarum_ extracellular polysaccharide. ECP of _R. salmoninarum_ F91 were digested with pronase for 24 h as described in the legend to Fig. 5, and samples equivalent to 30 μg of ECP protein prior to digestion were applied to the gels (lanes 1). Samples of undigested ECP, equivalent to 10 μg of ECP protein, are also shown (lanes 2). (A) The blot was subjected to periodate oxidation by the method of Woodward et al. (28). (B) The blot was subjected to the same procedure as that used for panel A except that periodate was omitted from the acetate buffer. The blots in both panels were developed with the polyclonal anti-pronase-digested cell lysate-boosted whole-cell antiserum.
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REFERENCES


FIG. 7. Western blot showing cross-reactivity of the polyclonal antiserum prepared against pronase-digested R. salmoninarum cell lyase-boosted whole-cell antiserum with O polysaccharides from A. salmonicida. Lipopolysaccharide fractions equivalent to 8 μg of outer membrane protein from A. salmonicida Asa 92 (lane 1), A. salmonicida Asa 35 (lane 2), P. fluorescens F (lane 3), and V. ordalii B (lane 4) or extracellular products equivalent to 100 μg of protein from R. salmoninarum F91 (lane 5) were subjected to Western blotting after digestion with pronase. Prestained molecular mass markers as described in the legend to Fig. 1 are shown in lane 6.

The ability of PMSF to inhibit proteolytic activity against gelatin in all of the p57 derivatives and of DTT to prevent this inhibition indicated that a common mechanism existed among the active polypeptides. Also, DTT appeared to activate ECP proteolytic activity against both an unrelated protein, BSA, and the autologous p57 protein and to be able to prevent the inhibition caused by PMSF. These results could be interpreted as being characteristics of a thiol proteinase (20). Inhibition of the ECP proteolytic activity by other sulfhydryl group-reactive inhibitors lends support to this interpretation. Accordingly, one reason for the variable detection of proteolytic bands in different ECP preparations by G-PAGE may be oxidation or inactivation of an enzyme relying on sulfhydryl groups for its activity. Also, this may explain the apparent stability of p57 isolated by isoelectric focusing (22), a procedure reported to inactivate thiol proteinases under nonreducing conditions (15).

During the course of these investigations, polysaccharide was detected as a major component of the R. salmoninarum ECP and in kidney tissue from Atlantic salmon clinically infected with the pathogen. The pronase-resistant, carbohydrate-positive series of bands migrating mainly below 40 kDa on Western blots of the ECP are characteristic of polysaccharide (19) and appeared to represent released capsular material reported to cover R. salmoninarum cells in addition to a surface-located fraction of p57 (5). Periodate oxidation (28) of this ECP material blocked its immunodetection on Western blots, which further confirmed its polysaccharide nature. Wood and Kaattari (26) reported that formalin-killed cells of R. salmoninarum, depleted of p57, produced enhanced antibody responses to cell surface carbohydrate moieties in chinook salmon (O. tshawytscha), suggesting the moieties were largely masked by p57. These immunogenic cell surface carbohydrate moieties may be representative of the polysaccharide material detected in the ECP. Cross-reaction of antiserum against the R. salmoninarum ECP polysaccharide with the O polysaccharides from A. salmonicida, an unrelated pathogen of salmonids, suggested some structural similarity in the polysaccharides which may be associated with common cellular receptors in host fish. Interestingly, it has been reported (18) that formalin-killed R. salmoninarum cells produced a potentiating effect in protecting coho salmon (O. kisutch), vaccinated with a formalin-killed A. salmonicida bacterin, against subsequent challenge with A. salmonicida.

The results presented here show that proteolytic activity is associated with p57 and several of its derivatives. Because these polypeptides are found in large concentrations in tissues of salmonids infected with R. salmoninarum (22) along with the extracellular polysaccharide, further studies on the potential roles of the proteolytic activity and the polysaccharide will be important to the eventual understanding of the pathogenesis of BKD infections and may lead to the development of potential vaccines.