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Effects of Short-Term Cold Storage on Recovery of Proteases from Extracellular Products of *Aeromonas hydrophila*

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Three protease-containing fractions were recovered by gel filtration from concentrated crude extracellular products produced by *Aeromonas hydrophila* grown in a defined medium. The recovery of a heat-stable protease was differentially prevented when the crude preparation was stored for 48 h at -20°C but was unaffected by storage of the crude preparation at either 4 or -70°C . Once fractionated, the heat-stable protease appeared to be unaffected by subsequent storage at 4, -20 , or -70°C .

Aeromonas hydrophila is an opportunistic pathogen that can cause extensive mortalities among aquacultured ectotherms (3, 4). The extracellular hemolysin(s) of this organism has been implicated as one of the virulence factors contributing to motile aeromonad septicemia in amphibians (7) and fish (1, 8). In addition, *A. hydrophila* has been reported to produce two proteases (1, 2, 9). One protease is labile, and the second is relatively stable after treatment for 10 min at 56°C (2, 9). Similar partially purified proteases have been implicated as lethal factors for young of the year channel catfish (9).

It has been suggested that the extracellular products (ECP) of *A. hydrophila* provide the common antigens necessary for the successful development of a vaccine against motile aeromonad septicemia (1). Our attempts to demonstrate common antigens among the proteases of strains of *A. hydrophila* have been equivocal. Among the considerations that could have an impact on such studies are the formation of complexes among components of the ECP (1, 5) and the possible destruction of individual components of the ECP by the proteases (1). The processing of large volumes of ECP usually requires short-term storage at some point, and the stability of the proteases in frozen samples of crude ECP has been reported to be variable (1). Therefore, we have evaluated the effects of short-term cold storage on the extracellular proteases of a hemolysin-deficient strain of *A. hydrophila*. The results of this study suggest that *A. hydrophila* produces three proteases, one of which appears to be masked but not lost by alterations caused by short-term storage of the crude ECP at -20°C .

With the exception that *A. hydrophila* 22 was subcultured for 5 months on agar slants of a defined medium (6), the procedures were as reported previously (9). Cell-free preparations from a 50-h batch culture, reduced from 10 liters to 200 ml by ultrafiltration, were fractionated with a Bio-Gel P-60 column (2.5 by 90 cm). Three protease-containing fractions (PI, PII, and PIII) were recovered when samples were fractionated immediately after ultrafiltration (Fig. 1). Similar elution profiles were reproduced after storage of the samples for 14 days at either 4 or -70°C . However, pronounced changes were observed in the elution profiles when portions of the same preparation were stored at -20°C .

Compared with the elution profile of PII, the equivalent fraction, PIIA, was substantially reduced after storage of the

crude ECP at -20°C for 24 h (Fig. 1). Furthermore, PIIA was essentially absent after storage of the crude ECP at -20°C for 48 h or longer. Total protein and protease determinations among the fractions suggested that the difference between the two elution profiles was not the result of PIIA destruction. It appeared that PIIA coeluted with PIA after the crude sample was stored at -20°C (Table 1). Analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis demonstrated that the changes in the crude ECP stored at -20°C were even more complex than those indicated by the Bio-Gel P-60 results. The bulk of the protein associated with PII migrated as two distinct bands with molecular weights in the range of 34,000 to 35,000 (Fig. 2). A similar band(s) was progressively lost from a crude sample stored at -20°C for 24 and 48 h. The loss of this material was consistent with the loss of PIIA in the gel filtration determinations (Fig. 1). Although no new bands were recovered from the frozen samples, other changes were observed (Fig. 2). A 32,000-molecular-weight band, a minor component in the PII banding pattern, did not appear to be markedly affected by storage for 24 h but was reduced after storage for 48 h at -20°C . Accompanying the reductions were increases in the density of three major bands with molecular weights of 56,000, 46,000, and 19,500. The 56,000- and 19,500-molecular-weight bands have been demonstrated to be the major components in fractions equivalent to PIA and PIIIA, respectively (9). The lower molecular weights that we originally reported for these two proteins resulted from an error in the labeling of the molecular weight standards that was subsequently identified and corrected.

Treatment for up to 50 min at 56°C demonstrated that PI and PIA proteases were labile, PII protease was stable with activity slightly stimulated, and, compared with the other proteases, PIII and PIIIA proteases were of intermediate stability (Fig. 3).

The increased intensity of staining of PI in the crude ECP polyacrylamide gel electrophoresis separations (Fig. 2) after storage at -20°C , the increased yield of protein and proteolytic activity in PI from fractions of material stored at -20°C (Table 1), and the concomitant loss of material from PII (Fig. 2 and Table 1) lead to speculation that molecular recombination has occurred and that the activity of PII has shifted to PI. However, under the electrophoretic conditions used, molecular recombination of subunits seems to be precluded. The similar rates of thermal inactivation between protease I and III fractions derived from freshly collected and frozen ECP (Fig. 3) indicate that the nature of the changes in PII

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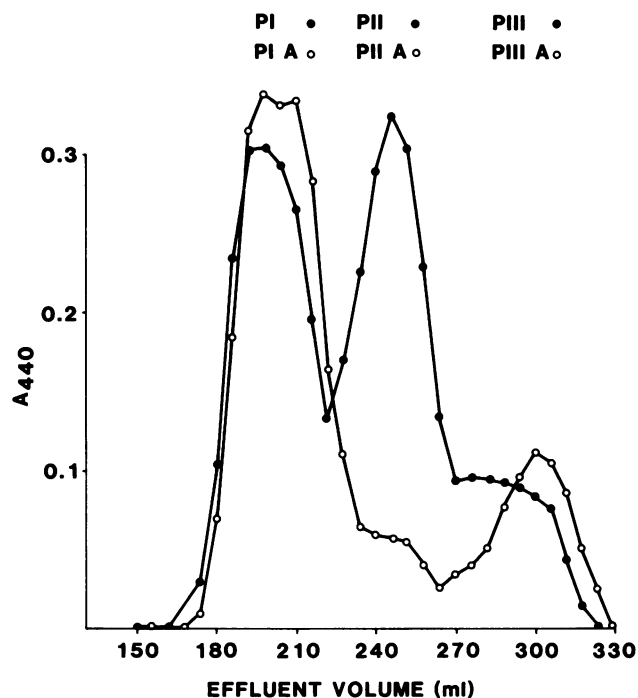


FIG. 1. Bio-Gel P-60 elution profiles of *A. hydrophila* 22 proteases from crude concentrated ECP freshly collected (●) or stored for 24 h at -20°C (○). The eluant was 0.05 M Tris-hydrochloride buffer (pH 7.0) with 0.25 M NaCl. Fractions (3 ml) were collected, and the original volume was 140 ml. Individual protease fractions are indicated. A_{440} , Absorbance at 440 nm.

either masks or destroys the heat stability of PII protease. Subsequent studies have shown that each of the three protease fractions from freshly collected ECP and reconstructed mixtures of these fractions, including those fractions that did not contain protease, were unaffected after storage at the three temperatures (unpublished data). Thus, the changes associated with storage at -20°C appear to be confined to the intact crude ECP.

Our initial study used *A. hydrophila* ECP stored at -20°C (9). Dahle (2) incorporated a -20°C treatment step in his preparation of the crude ECP from *Aeromonas liquefaciens* (*A. hydrophila*), but Allan and Stevenson (1) stored their ECP at 4°C . All three studies demonstrated two proteases by gel filtration techniques, but obvious differences in experimental protocols and insufficient information do not permit direct comparisons among the studies. Both Dahle (2) and Allan and Stevenson (1) harvested their ECP from skim milk

TABLE 1. Distribution of total protein and protease activity among Bio-Gel P-60 fractions from the crude concentrated ECP of *A. hydrophila* 22

Fraction	Protein (mg)	Protease (U)
PI ^a	25.0	255
PII	4.6	184
PIII	10.3	203
PIA ^b	30.6	400
PIIA	0.7	5
PIIIA	9.0	211

^a From a freshly collected crude sample.

^b From a portion of the same sample stored for 48 h at -20°C .

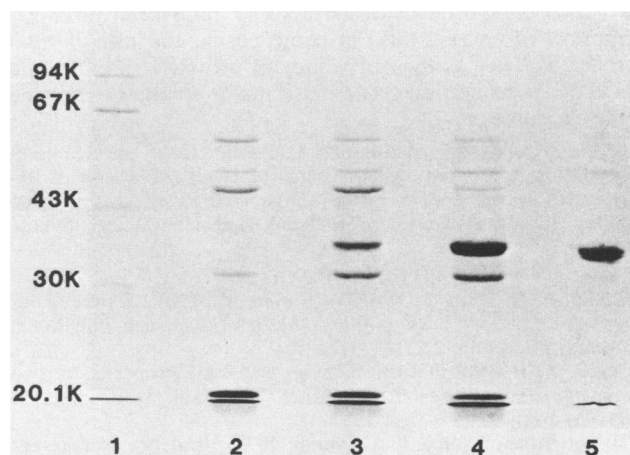


FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of *A. hydrophila* 22 preparations. Lane 1: Standard proteins and their molecular weights. From top, phosphorylase *b* (94,000), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), and trypsin inhibitor (20,100). Lane 2: Crude ECP stored at -20°C for 48 h. Lane 3: Crude ECP stored at -20°C for 24 h. Lane 4: Freshly collected crude ECP. Lane 5: Bio-Gel P-60 protease fraction (PII) from freshly collected crude ECP. All samples were heated (100°C for 5 min) and reduced (2-mercaptoethanol, 5%).

agar cultures. Achieved by using a defined medium and larger volumes of ECP produced in a fermentor, our results suggest that at least one strain of *A. hydrophila* can produce three extracellular proteases. Storage of the crude concentrated ECP of this isolate at -20°C for 24 to 48 h appears to differentially alter the recovery of a heat-stable protease component by a number of techniques common to the initial studies on the isolation, purification, and characterization of enzymes. However, these alterations did not appear to be at

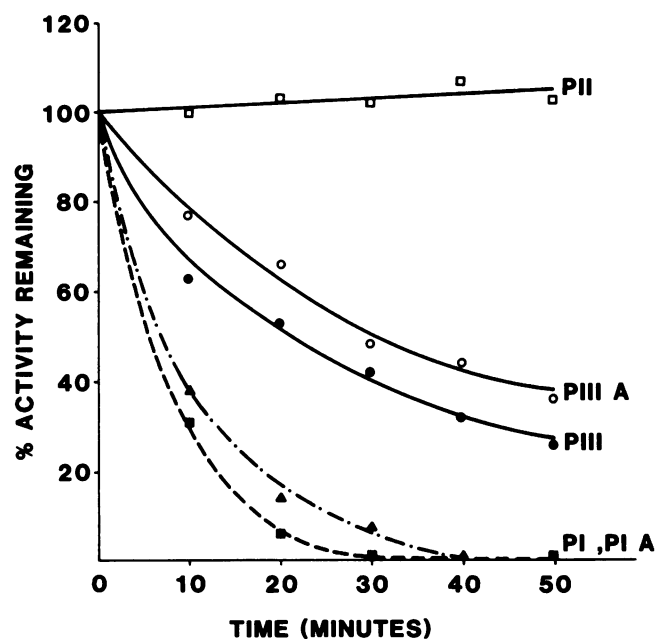


FIG. 3. Heat stability (56°C) of the protease activity of *A. hydrophila* 22 Bio-Gel P-60 fractions from freshly collected ECP (PI, PII, and PIII) and ECP stored at -20°C for 48 h (PIA and PIIIA).

the expense of total protease activity recovered after gel filtration (Table 1). Thus, in some cases, the use of total protease activity as the only index of protease stability in a crude ECP preparation could mask major changes occurring in the preparation.

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