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In Vitro Proteolytic Processing and Activation of the Recombinant Precursor of El Tor Cytolysin/Hemolysin (Pro-HlyA) of *Vibrio cholerae* by Soluble Hemagglutinin/Protease of *V. cholerae*, Trypsin, and Other Proteases

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Vibrio cholerae **produces a cytolytic toxin named El Tor cytolysin/hemolysin which is encoded by the *hlyA* gene. This cytolysin is produced as a 79-kDa precursor form (pro-HlyA) into the culture supernatant after cleavage of the signal peptide of the *hlyA* product (prepro-HlyA). The pro-HlyA is then processed to a 65-kDa mature cytolysin (mature HlyA) after cleavage of the 15-kDa N-terminal peptide (pro region) of the 79-kDa precursor, usually at the bond between Ala-157 and Asn-158. We investigated whether proteases could process the recombinant 79-kDa pro-HlyA to the 65-kDa mature HlyA. We observed that the soluble hemagglutinin/protease (HA/protease; a major protease of *V. cholerae*), trypsin, α-chymotrypsin, subtilisin BPN′, papain, and thermolysin all processed the pro-HlyA to the 65-kDa mature form of the protein. Along with this, the protease-processed HlyA showed drastically increased hemolytic activity. The N-terminal amino acid of the mature form of cytolysin generated by HA/protease was Phe-151, and that due to trypsin was Ser-149. Other proteases also cleaved the pro-HlyA at a nearby site, between Leu-146 and Ser-153, and all the processed cytolysins showed increased hemolytic activity. These data suggest that the active El Tor cytolysin of *V. cholerae* could be derived from the C-terminal region of a pro-HlyA following proteolytic cleavage of the bonds in the vicinity of Leu-146 to Asn-158 by any of a wide variety of proteases.**

The El Tor biotype of *Vibrio cholerae* O1 is the causative agent of the current, seventh cholera pandemic. Unlike classical-biotype strains, El Tor biotype strains and most of *V. cholerae* non-O1 strains produce a cytolytic toxin (El Tor cytolysin/hemolysin) and secrete it into the culture medium (9, 18, 19). This feature has been used as a marker to distinguish between the two biotypes. The enterotoxic activity of the purified cytolysin suggests that the cytolysin may contribute to the pathogenesis of gastroenteritis that is caused by *V. cholerae* strains lacking the cholera toxin gene (10).

A cytolysin purified from the culture supernatant of an El Tor biotype strain, N86, had an apparent molecular mass of 60 to 65 kDa (mature HlyA) (19), although the predicted molecular size of the product of the cloned gene was around 82 kDa (prepro-HlyA) (20). We had previously detected a 79-kDa precursor form (pro-HlyA) in the supernatants of earlier cultivation stages by Western blotting (immunoblotting) and pulse-chase analysis (20). The results of sequencing the N-terminal amino acids of the 79-kDa pro-HlyA and the mature HlyA suggested that the 82-kDa prepro-HlyA is processed into 79-kDa pro-HlyA by release of the signal peptide of 25 amino acid residues and is then excreted into the supernatant through the outer membrane (20). The 79-kDa pro-HlyA has been found to be further processed into 65-kDa mature HlyA in culture supernatants following cleavage of the N-terminal 15-kDa polypeptide of the pro-HlyA (pro region). This cleaving process that turns pro-HlyA into mature HlyA is thought to occur via extracellular proteolysis, but the protease or proteases which are responsible have not been determined.

Soluble hemagglutinin of *V. cholerae* has been purified as a candidate for the colonization factor (7) and was determined to have several other biological activities besides its agglutination effect on erythrocytes; they include proteolytic activity and lectin-like activity (5). The soluble hemagglutinin/protease (HA/protease) is a major extracellular proteolytic activity of *V. cholerae* (8, 21). Consequently, in this study, we attempted to process the pro-HlyA with the HA/protease and also with trypsin, which is a common protease in the small intestine. We demonstrated that purified *V. cholerae* HA/protease processes the recombinant 79-kDa pro-HlyA to the 65-kDa cytolysin, with an accompanying activation of hemolytic activity. The results also suggest that trypsin and other proteases process the pro-HlyA to the mature, active form of the cytolysin.

MATERIALS AND METHODS

Purification of the recombinant 79-kDa precursor. Although we had previously attempted to purify the 79-kDa precursor from the supernatant of the *V. cholerae* culture, the purification was unsuccessful because of the unstability of the crude precursor protein during the purification process. Consequently, the 79-kDa HlyA product expressed in *Escherichia coli* was used as the precursor of the El Tor cytolysin. To overexpress the HlyA precursor protein (pro-HlyA) in *E. coli*, we used a recombinant plasmid, pKY156, which had a DNA fragment containing the coding region of the *hlyA* gene in an expression vector, pKK223-3, as previously reported (20).

The 79-kDa pro-HlyA was expressed in *E. coli* JM109 by induction with isopropyl-β-D-thiogalactopyranoside (IPTG) during the exponential growth phase. The precursor was purified by column chromatography from the cell extract as previously described (20). The N terminus of the purified precursor protein was confirmed to be Asn-26, indicating that the signal peptide had been removed.

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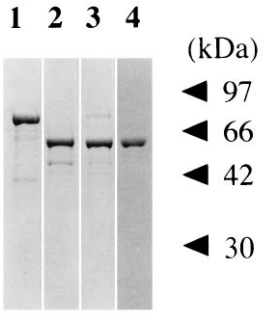

FIG. 1. SDS-PAGE of HA/protease- or trypsin-treated HlyA. The sample treated with protease (0.8 volume) was incubated with 0.2 volume of 5× SDS-PAGE sample solution (0.1% Coomassie brilliant blue, 1% SDS, 5% 2-mercaptoethanol) for 5 min at 95°C. The mixture was subjected to SDS–10% PAGE, and the gels were stained with Coomassie brilliant blue. Lanes: 1, pro-HlyA; 2, trypsin-treated HlyA; 3, HA/protease-treated HlyA; 4, mature HlyA.

Purification of mature El Tor cytolysin. Mature El Tor cytolysin (mature HlyA) was purified from the supernatant of a 48-h culture of *V. cholerae* O1 N86 as previously reported (19).

Purification of soluble HA/protease. Soluble HA/protease was purified from *V. cholerae* non-O1 TH81 by ammonium sulfate precipitation and immunoaffinity column chromatography as previously described (15).

Other proteases. All other proteases used in this study—trypsin, α-chymotrypsin, thermolysin, subtilisin BPN′, and papain—were purchased from Sigma (St. Louis, Mo.).

Protease treatments. Phosphate-buffered saline (PBS), pH 7.0, containing 9 nmol of pro-HlyA per ml was mixed with an equal volume of protease in the same buffer and incubated at 37°C for 30 min. The protease-treated samples were immediately subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and hemolytic assay. The molar ratios of HA/protease, trypsin, α-chymotrypsin, subtilisin BPN′, papain, and thermolysin to pro-HlyA were 1/100, 1/10,000, 1/1,000, 1/10,000, 1/1,000 and 1/1,000, respectively.

SDS-PAGE. Electrophoresis of proteins was carried out by the method described by Laemmli (12), using 10% polyacrylamide gel containing 0.1% SDS. Gels were stained with Coomassie brilliant blue R-250 (Sigma).

Protein content. Protein content was determined by the method of Lowry et al. (13) with bovine serum albumin as the standard.

Hemolysis assays. Assays for hemolytic activity of pro-HlyA and protease-treated HlyA protein were carried out against sheep erythrocytes as previously described (17). Briefly, sheep erythrocytes were washed in PBS three times and adjusted to a hematocrit of 2% in PBS. Samples were serially diluted with PBS and immediately mixed with an equal volume (60 μl) of the erythrocyte suspension. The reaction mixtures were kept at 37°C for 2 h. After centrifugation at 2,000 × *g* for 30 s, aliquots of the supernatants (100 μl) of the reaction mixtures were placed in a 96-well plate for spectrophotometric measurement at 540 nm with the Multiscan MCC/340 (Labosystems, Tokyo, Japan). In the results, 1 hemolytic unit (HLU) is defined as the activity required to lyse 50% of the cells in 0.1 ml of 1% sheep erythrocytes in PBS at 37°C in 2 h.

Amino acid sequencing. Sequences of N-terminal amino acids were determined by Edman degradation with an automatic protein sequencer (model 473A or 492; Applied Biosystems, Foster City, Calif.). Samples were subjected to SDS-PAGE, electrophoretically transferred to a polyvinylidene difluoride membrane (Immobilon; Millipore, Bedford, Mass.), and stained with Coomasie brilliant blue, and the 65-kDa protein bands were extracted from the polyvinylidene difluoride membrane before sequencing.

RESULTS

Processing of pro-HlyA by HA/protease and trypsin. It was predicted that the major secreted protease of *V. cholerae*, HA/protease, which is believed to activate cholera enterotoxin (2, 4), may initiate the conversion of pro-HlyA to mature HlyA. Consequently, we attempted to generate mature cytolysin by digestion of the pro-HlyA with the protease. When pro-HlyA was treated with HA/protease, the molecular size was reduced from 79 kDa (Fig. 1, lane 1) to 65 kDa (lane 3), which is identical to the size of the purified mature cytolysin from *V. cholerae* culture supernatant (lane 4). This suggests that the N-terminal pro region of pro-HlyA may be cleaved off by treatment with HA/protease, in a manner similar to the processing of mature HlyA in the culture supernatant of *V. cholerae* (20). We also tested trypsin to determine if it could process pro-HlyA to mature HlyA, since this protease is a major digestive enzyme in the small intestine. When the pro-HlyA was treated with trypsin, the protein was processed to the 65-kDa form (lane 2), which is identical in molecular size to HA/protease-treated HlyA (lane 3).

Cleavage sites. To reveal the exact cleavage sites of HA/protease- and trypsin-treated HlyA for comparison with the sites of cleavage in native mature HlyA, the N-terminal amino acid sequences were determined. Although the N-terminal amino acid of the native mature HlyA is Asn-158, the first 10 N-terminal amino acids of HA/protease- and trypsin-treated HlyAs were determined to be Phe-151 to Glu-160 and Ser-149 to Asn-158, respectively, corresponding to the deduced amino acid sequence of HlyA (20). This indicates that HA/protease and trypsin cleave the peptide junctions seven and nine residues upstream, respectively, of the site of cleavage in the native cytolysin purified from *V. cholerae* supernatant. This suggests that the actual processing enzyme of *V. cholerae* N86 for the pro-HlyA may not be the HA/protease.

Activation of pro-HlyA with processing. Previously, we found that the specific activity of the 65-kDa mature HlyA was much higher than that of the 79-kDa pro-HlyA (20). In that study, however, we compared the mature HlyA from *V. cholerae* and the pro-HlyA from *E. coli* which had expressed the *hlyA* gene product. Thus, the difference in the activities of the pro-HlyA and the mature HlyA may have been due to the difference in host species. To investigate whether the enhanced activity of mature HlyA is due to the release of the pro region from pro-HlyA rather than the host bacterium used for expression of the gene, we compared the specific activity of pro-HlyA with that of protease-treated HlyA, which lacks the pro region. The specific activity of the pro-HlyA was 9 HLU/nmol, whereas those of HA/protease- and trypsin-treated HlyAs, which showed the same level of activity as the mature cytolysin purified from *V. cholerae* (600 HLU/nmol), were 580 and 650 HLU/nmol, respectively. This indicates that HA/protease and trypsin activate the pro-HlyA, simultaneously with the removal of the N-terminal 15-kDa pro region, and that the higher activity of the mature HlyA in the previous report (20) had been due to the processing of the pro region rather than to the difference in host species. After a longer period (60 min) of incubation with proteases, however, the cytolytic activity began to decrease; this was likely due to the further degradation of the active enzyme forms by overdigestion with the proteases (data not shown).

Processing and activation of pro-HlyA by other proteases. Although HA/protease removed the pro region of pro-HlyA from the mature region, this enzyme is unlike the native processing enzyme of *V. cholerae* for El Tor cytolysin, since the cleavage position (Gly-150–Phe-151) was different from that of the native mature HlyA (Ala-157–Asn-158). This suggests that proteolytic enzymes other than HA/protease may process and activate pro-HlyA in the culture supernatant of *V. cholerae*. We attempted to investigate what kind of proteases may possess this specific activity. We investigated four different proteases, namely, α-chymotrypsin (a trypsin-like serine protease), subtilisin BPN′ (a serine protease, but different from the trypsin group proteases), papain (a cysteine protease), and thermolysin (a metalloprotease) (16). When we used these proteases to treat pro-HlyA, all four reduced the molecular mass from 79 kDa (Fig. 2, lane 1) to 65 kDa (lanes 3 to 6), the same molecular size as mature HlyA (lane 7) and the processed pro-HlyA treated with HA/protease or trypsin (Fig. 1; Fig. 2, lane 2).

To locate the exact cleavage sites of HlyA proteins treated

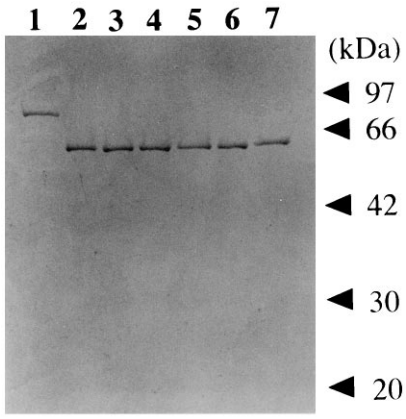

FIG. 2. SDS-PAGE of protease-treated HlyA. Lanes: 1, pro-HlyA; 2, trypsin-treated HlyA; 3, α-chymotrypsin-treated HlyA; 4, subtilisin BPN′-treated HlyA; 5, papain-treated HlyA; 6, thermolysin-treated HlyA; 7, mature HlyA.

with these proteases, the N-terminal amino acid sequences were determined. The N-terminal amino acids of pro-HlyA treated with α-chymotrypsin, subtilisin BPN′, papain, and thermolysin were Ala-152, Ala-147, Ser-153, and Leu-146, respectively (Fig. 3). These results suggest that a variety of types of protease are able to process the pro-HlyA from 79 kDa to the 65-kDa mature protein by cutting in the vicinity of the native processing site (Asn-158).

We further investigated whether the activation of the pro-HlyA might accompany the removal of the pro region by these proteases, in a manner similar to HA/protease and trypsin. Pro-HlyA was treated with these proteases individually, and hemolytic activities were determined (Fig. 4). The resultant hemolytic activities were 44 to 90 times higher than that of untreated pro-HlyA, similar to the situation with trypsin-treated HlyA and mature HlyA. These results suggest that these proteases not only cleave in the vicinity of the native processing point of pro-HlyA but also increase the hemolytic activity of pro-HlyA. Furthermore, these data indicate that exact cleavage at the Ala-157–Asn-158 bond is not always essential but that cleavage at that site or any one of several N-terminal amino acid residues downstream from it (Leu-146 to Asn-158) activates pro-HlyA.

DISCUSSION

Previously, we reported that the purified mature cytolysin from *V. cholerae* lacked the 15-kDa N-terminal sequence (pro region) and that the cytolytic activity was much higher than that of the recombinant precursor (pro-HlyA) (20). The simplest explanation for this observation is that the cytolysin was

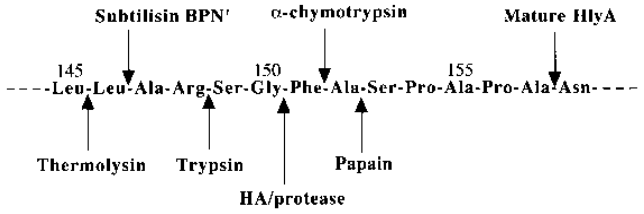

FIG. 3. Cleavage positions of protease-treated HlyA and mature HlyA. A total of 10 to 15 amino acid sequences of protease-treated HlyAs and mature HlyA were determined. The first amino acid (Met) deduced from the *hlyA* gene was designated as 1, and 14 amino acids between Leu-145 and Asn-158 were indicated.

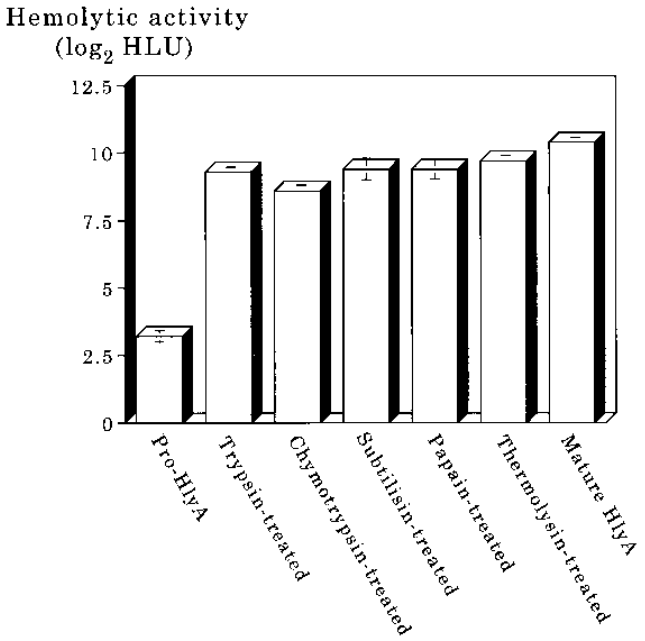

FIG. 4. Activation of hemolytic activity of pro-HlyA by protease treatment. Pro-HlyA was treated with protease and the hemolytic activity determined as described in Materials and Methods.

activated by removal of the pro region. We speculated that the activity of the cytolysin might be posttranslationally regulated by a proteolytic enzyme produced by the organism. We suspected that one of the possible processing enzymes for El Tor cytolysin might be HA/protease, because this enzyme is a major extracellular protease of *V. cholerae* and plays a role in the activation of cholera toxin (2, 4). Here we have demonstrated that HA/protease does process the 79-kDa recombinant pro-HlyA to the 65-kDa mature form of the cytolysin. The processed protein has a molecular size similar to that of the mature cytolysin of *V. cholerae*. Subsequent to the processing, the cytolytic activity increases more than 40 times, which is as active as the mature HlyA purified from *V. cholerae*. We also found that trypsin and some other proteases can remove the pro region from the pro-HlyA to generate the 65-kDa protein, resulting in similar increases in the cytolytic activity. These findings suggest that in a *V. cholerae* culture, the 65-kDa El Tor cytolysin may be derived from a weakly active single-chain precursor (pro-HlyA) subsequent to proteolytic cleavage at (or near) the Ala-157–Asn-158 bond (20).

The cleavage site of HA/protease-processed HlyA, however, was Gly-150–Phe-151, whereas the site of cleavage in the culture supernatant of *V. cholerae* N86 was the Ala-157–Asn-158 bond (20). This seems to rule out a role for HA/protease in the processing of El Tor cytolysin and suggests another, unknown processing enzyme. As yet, we have not identified the protease which cleaves the Ala-157–Asn-158 bond of the pro-HlyA in *V. cholerae* N86. The cleavage site at the Gly-150–Phe-151 bond, however, has been reported to generate the 65-kDa mature HlyA for *V. cholerae* 8731 (6), suggesting that HA/protease may work as a processing and activating enzyme in some strains other than N86.

The processing and activation of the cytolysin precursor by trypsin, which is abundant in the small intestine, may occur at the site of infection. This enzyme may presumably play a role in diarrhea due to cytolysin produced in vivo in food poisoning

by *V. cholerae* El Tor and non-O1 strains which do not produce cholera enterotoxin. Some of the clinical studies of diarrhea due to non-O1 *V. cholerae* strains reported hemorrhagic diarrhea as a clinical feature (1, 18). These reports indicate the prominence of cytotoxicity in cholera, which may be due to El Tor cytolysin.

HA/protease cleaves at the Gly-150–Phe-151 bond, trypsin cleaves at the Arg-148–Ser-149 bond, and the other proteases cleave in the region between Leu-146 and Ser-153, accompanied by increasing hemolytic activity. This suggests that cleavage precisely at Ala-157–Asn-158, as seen for the culture supernatant of *V. cholerae* N86 (20) and S7 (11), is by no means essential for the activation of pro-HlyA. Many other types of proteases are, in fact, capable of activating pro-HlyA—for example, a trypsin-like serine protease (α-chymotrypsin), a serine protease different from trypsin (subtilisin BPN′), a cysteine protease (papain), and a metalloprotease (thermolysin) (Fig. 4). Compared with these proteases, trypsin presents advantages for analysis of the phenomenon because trypsin is a relatively stable enzyme that is easy to obtain commercially; it also has well-restricted cleavage sites.

V. cholerae O1 strain 8731 has been reported to cleave at the Gly-150–Phe-151 bond, in a manner similar to HA/protease-treated HlyA (6). *V. cholerae* non-O1 strain N037 produces a smaller cytolysin (48 kDa), although it has a much lower specific activity than that of the 65-kDa native cytolysin (11). As the N-terminal residue of the 48-kDa cytolysin (Ala-155) is very close to that of the 65-kDa cytolysin (Asn-158), the 48-kDa cytolysin is considered to be truncated at the C terminus. On the other hand, the pro-HlyA of *V. cholerae* non-O1 strain S7 was cleaved at a site identical to that of the N86 cytolysin (11). The cultivation conditions for S7 were the same as those for N86 (18, 19). We speculate that the variety of cleavage sites reported may be due to different proteases which arise under different culture conditions and that these conditions reflect the proteolytic degradation of cytolysin and the total recovery of cytolysin of *V. cholerae*. Our results and these reports suggest that the region around Leu-146 to Asn-158 might be very sensitive to many kinds of proteases; consequently, for full activation of the cytolysin, cleavage at or near the Ala-157–Asn-158 junction is essential.

We have observed that pro-region-deleted mutant HlyA is not secreted and degraded in the periplasmic space; guanidine-HCl-denatured pro-HlyA fully recovers the activity by reducing the concentration of the denaturant, but mature-HlyA, which lacks the pro region, does not recover the activity (14). These results indicate that the role of the pro region could be to fold the cytolysin itself. We still do not, however, know the relationship between the release of the pro region and the activation of cytolysin. In this regard, it could be speculated that the pro region of HlyA might hinder oligomerization to form a pore, which is necessary for the cytolytic activities of many kind of hemolysins (3). The biological significance of proteolytic activation of the cytolysin is also unclear, but the pro region might be a safety lock for bacterial cells, a place which restrains the cytolytic potential, which would be toxic to the *V. cholerae* cell itself if it were active inside the bacterial cell.

The data presented here suggest that proteolytically enhanced processing of pro-HlyA is an obligatory step in the activation of El Tor cytolysin and that cleavage around Leu-146 to Asn-158 is likely to trigger the activation of the pro-HlyA. The exact range in which processing will activate the pro-HlyA is still unclear, and it also unknown which protease activates pro-HlyA cleavage at the native processing site, Ala-157–Asn-158. Moreover, the structural consequences of the processing event also need to be determined—for example, the changes in the circular dichroism and infrared spectra and other biophysical measurements. Further study is needed to gain an understanding of the molecular mechanism involved in the processing and activation of El Tor cytolysin. The first step in this undertaking, the identification of the native processing enzyme of El Tor cytolysin, is underway. We think this report will shed some light on the posttranslational modification of the protein.

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REFERENCES

1. **Blake, P. A.** 1980. Diseases of humans (other than cholera) caused by vibrios. Annu. Rev. Microbiol. **34:**341–367.
2. **Booth, B. A., M. Boesman-Finkelstein, and R. A. Finkelstein.** 1984. *Vibrio cholerae* hemagglutinin/protease nicks cholera enterotoxin. Infect. Immun. **45:**558–560.
3. **Braun, V., R. Schonherr, and S. Hobbie.** 1993. Enterobacterial hemolysins: activation, secretion and pore formation. Trends Microbiol. **1:**211–216.
4. **Finkelstein, R. A., M. B. Finkelstein, and P. Holt.** 1983. *Vibrio cholerae* hemagglutinin/lectin/protease hydrolyzes fibronectin and ovomucin: F. M. Burnet revisited. Proc. Natl. Acad. Sci. USA **80:**1092–1095.
5. **Finkelstein, R. A., and L. F. Hanne.** 1982. Purification and characterization of the soluble hemagglutinin (cholera lectin) produced by *Vibrio cholerae*. Infect. Immun. **36:**1199–1208.
6. **Hall, R. H., and B. S. Drasar.** 1990. *Vibrio cholerae* HlyA hemolysin is processed by proteolysis. Infect. Immun. **58:**3375–3379.
7. **Hanne, L. F., and R. A. Finkelstein.** 1982. Characterization and distribution of the hemagglutinins produced by *Vibrio cholerae*. Infect. Immun. **36:**209–214.
8. **Häse, C. C., and R. A. Finkelstein.** 1991. Cloning and nucleotide sequence of the *Vibrio cholerae* hemagglutinin/protease (HA/protease) gene and construction of an HA/protease-negative strain. J. Bacteriol. **173:**3311–3317.
9. **Honda, T., and R. A. Finkelstein.** 1979. Purification and characterization of a hemolysin produced by *Vibrio cholerae* biotype El Tor: another toxic substance produced by cholera vibrios. Infect. Immun. **26:**1020–1027.
10. **Ichinose, Y., K. Yamamoto, N. Nakasone, M. J. Tanabe, T. Takeda, T. Miwatani, and M. Iwanaga.** 1987. Enterotoxicity of El Tor-like hemolysin of non-O1 *Vibrio cholerae*. Infect. Immun. **55:**1090–1093.
11. **Iwanaga, M., and Y. Ichinose.** 1991. An aberrant hemolysin of *Vibrio cholerae* non-O1. Microbiol. Immunol. **35:**705–715.
12. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
13. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
14. **Nagamune, K., K. Yamamoto, and T. Honda.** Submitted for publication.
15. **Naka, A., T. Honda, and T. Miwatani.** 1992. A simple purification method of *Vibrio cholerae* non-O1 hemagglutinin/protease by immunoaffinity column chromatography using a monoclonal antibody. Microbiol. Immunol.**36:**419–423.
16. **Neurath, H.** 1984. Evolution of proteolytic enzymes. Science **224:**350–357.
17. **Tang, G.-Q., T. Iida, K. Yamamoto, and T. Honda.** 1994. A mutant toxin of *Vibrio parahaemolyticus* thermostable direct hemolysin which has lost hemolytic activity but retains ability to bind to erythrocytes. Infect. Immun. **62:** 3299–3304.
18. **Yamamoto, K., M. Al-Omani, T. Honda, Y. Takeda, and T. Miwatani.** 1984. Non-O1 *Vibrio cholerae* hemolysin: purification, partial characterization, and immunological relatedness to El Tor hemolysin. Infect. Immun. **45:**192–196.
19. **Yamamoto, K., Y. Ichinose, N. Nakasone, M. Tanabe, M. Nagahama, J. Sakurai, and M. Iwanaga.** 1986. Identity of hemolysins produced by *Vibrio cholerae* non-O1 and *Vibrio cholerae* O1, biotype El Tor. Infect. Immun. **51:**927–931.
20. **Yamamoto, K., Y. Ichinose, H. Shinagawa, K. Makino, A. Nakata, M. Iwanaga, T. Honda, and T. Miwatani.** 1990. Two-step processing for activation of the cytolysin/hemolysin of *Vibrio cholerae* O1 biotype El Tor: nucleotide sequence of the structural gene (*hlyA*) and characterization of the processed products. Infect. Immun. **58:**4106–4116.
21. **Young, D. B., and D. A. Broadbent.** 1982. Biochemical characterization of extracellular proteases from *Vibrio cholerae*. Infect. Immun. **37:**875–883.

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